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# Microbial interactions with Diazinon.

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# MICROBIAL INTERACTIONS WITH DIAZINON

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# INTRODUCTION

Twenty-five years ago, Dr. Paul Mueller, conducting basic studies on a compound discovered at the turn of the century, demonstrated that DDT had insecticidal properties. For his work he received the Nobel Prize in Medicine and Physiology in 1948. In the intervening years, the use of modern synthetic pesticides has grown to the staggering level of 175 thousand tons a year as of 1962, and more is being used annually.

The need for such levels of pesticides is clearly shown when the following statistic is considered: 28% of all foodstuffs is lost between sowing and retail purchase due to weeds, fungi, insects, and rodents (11). If pesticides were not used, losses would be even greater; on the other hand, with increased applications of pesticides, the loss could be further reduced. With the application of such vast quantities of pesticides, the problem of pesticide residue has arisen, first called to public attention by Rachel Carson in her book Silent Spring (16).

There are many chemical events that may overtake a pesticide molecule once it reaches the soil; what, in fact, does happen is but poorly understood. What has emerged, however, is that as the last link of the food chain, the average American adult accumulates 100 to 200 mg DDT in liver fatty tissues (61). This, then, defines the problem: to account for the fate of pesticide molecules once they

reach the soil.

This paper reports an investigation on the influence of one representative organophosphate pesticide, Diazinon, on the soil microflora and the metabolism of one of its major hydrolysis products.

# LITERATURE REVIEW

A man died after drinking in a tavern that had recently been sprayed for cockroaches; his death was caused by toxic effects of the pesticide used in the spray (22). A massive fish kill in the Mississippi River followed increased levels of pesticides applied to fields bordering the river. Following an application of DDT at 1/50 ppm to kill gnats in a lake, herbivorous fish ate plankton that had absorbed the chemical. Carnivores ate the herbivores; birds ate the larger carnivores and herbivores and subsequently died, having concentrated 1,600 ppm DDT in their bodies (16). Pesticides cause degenerative changes in the liver and kidney; they cause nervous disorders, decrease avian fertility, cause blindness in fish, uncouple the vital oxidative phosphorylation, and disrupt enzymic reactions in the oxidative cycle (16). Pesticides have been detected in many food items, in clothing, in man and animals, and in various parts of the natural surroundings (61). Nonetheless, in spite of such results of pesticide application, these compounds are not only tolerated, but in a world threatened by starvation, their expanded use is mandatory.

Today it is estimated that 20% of all foodstuffs are lost between sowing and harvesting due to weeds, fungi, and insects; 10% of all food harvested is lost during transport and in storage due to rodent, insect, and fungus attack (11).

In a study of crop yields in gardens treated and untreated with pesticides, the pound yield for the treated areas was: for cabbage, 3.6 times that of the untreated areas; for snap beans, 2.8 times; cucumbers, 1.8; tomatoes, 1.1; and for potatoes, 1.2 times (24).

In 1947, 124.25 million pounds of synthetic pesticide was produced; in 1960, production reached the level of 637.7 million pounds at a wholesale value of one-quarter billion dollars (16). In 1962, 350 million pounds of insecticide alone were used (61); about 5% of the total land of the United States is today treated with an average of 2.5 pounds per acre of insecticide each year (54). Thus, there is a firm and growing commitment to the use of pesticides, which must now be met by an equally vigorous program of research. At the same time, it must be admitted that the benefits of pesticide application are not all good. There is a need to establish precisely the effect of pesticides on each of the members of the food chain, as a basis for understanding the influence of pesticides on human health and the natural populations in general (72).

With such great amounts of insecticide being applied yearly to soil, there is already a base of research on the fate of the pesticide molecule. Breakdown of insecticides has been studied in plants, insects, mammals, and, to a lesser extent, in the soil. It is known that the chlorinated hydrocarbon insecticides are resistant to significant

breakdown, whereas organic phosphorous compounds are more rapidly degraded; though sometimes they have persisted from one growing season to the next (61). Pesticide metabolism has been widely studied. It has been reported to occur in plants (18,27,48,62), in insects and mammals (18,42,68), and in the soil by the microflora or by isolates from the soil (2,9,33,38,43,44).

It is already clear that not all pesticide molecules are metabolized or are capable of being metabolized. Once the pesticide molecule reaches the soil, it can be bound by clay particles which attract phosphorous and nitrogen-containing compounds (4) or by soil colloids (40); once bound, these substances are not available to biological attack. Pesticides can be washed down below the surface soils into the ground water or carried in runoffs to nearby streams, causing injury to avian and aquatic life.

The interaction of pesticides and the environment affects both; pesticide molecules in the process of being decomposed also exert influences on their surroundings. An herbicide, 2,4-D, causes growth, though abnormal, in target plants (77); cotoran, another herbicide, when taken up by algae, disrupts the light-dependent portion of chlorophyl and/or chloroplast synthesis (67). The insecticides have a decided influence on the insect population, provided, that is, resistance has not been developed. An extensive amount of work has been done on insecticides and the development

and mechanism of resistance in insects (12,15,20,32,42,49, 56,58,59,74,75). To mammals, many of the insecticides are toxic, if not fatal; one group of insecticides, the organophosphates, exhibit from weak to very strong anticholinesterase activity; other types of insecticides are capable of attacking different mammalian enzyme systems. This area has also received much experimental attention (39,42,59,64, 68).

The work done on the effects of pesticides in general and insecticides in particular, referred to above, has been conducted in familiar systems -- plants, insects, mammals -- systems which have received experimental attention for many years. There is yet another area which, only in the last few years, has received much attention, the area of the soil microflora. The vast complexity of the ecology of soil microfloral communities has impeded studies in this area.

Nevertheless, a body of knowledge on pesticidal influence on the soil and its microflora has begun to emerge. When certain pesticides are applied to the soil, the supply of soluble plant nutrient elements increases through decomposition of dead material killed by pesticides and increased soil organic matter arising from increased microbial activity (46). There appears to be evidence that pesticides may exert direct chemical action on the soil, increasing soluble calcium, manganese, and other elements (46). It has been reported that Parathion, an organophos-

phorus insecticide, may exert a stimulating effect on some soil microflora, especially at 17.7% moisture holding capacity (52). Organophosphate insecticides, when applied to soils, induced an initial increase and subsequent decrease in soil $CO_2$ production, the degree of effect related to the concentration of insecticide applied (8). When Parathion, at low concentrations, was applied to soil, the total microbial population, the nitrogen fixers, nitrifiers, cellulose decomposers, and anaerobes were found to have increased in numbers. At slightly higher application levels, the Parathion concentration, 3 weeks following application, was 1/10 the original amount (53). There is evidence that other organophosphate insecticides exhibit a stimulatory effect on soil microflora (66). Pestox is reported to stimulate the microflora up to a concentration of 3,000 ppm (9).

Build-up of insecticide is possible in fields that receive multiple treatments each year and support growth of the same crop, year after year. In a study of such a process, cotton fields, cultivated with cotton annually, received the equivalent of 72 applications of pesticide in 3 years; a survey of the fields indicated decreasing levels of all four insecticides applied from a year following the initial and heaviest insecticide application. When compared with the total amount of insecticide applied, the detected levels at the end of the experiment were: DDT

levels, 1/3; BHC, 1/20, Guthion and Malathion levels were barely detectable (63).

Not all insecticides are removed from the soil by microbial activity. Such was the case for a study involving Aldrin, in which it was found that Aldrin losses from soil were due to other than microbial activity (28). It begins to appear, therefore, that once the pesticide has appeared in an environment, a dynamic relationship is established between the pesticide molecules and the biological systems present. In this relationship, neither party remains unchanged. The degree of alteration of both parties depends upon the extent to which they interact.

In the development of pesticides, insecticides in particular, one of the properties looked for was disappearance in the soil, in spite of prolonged application. Chlorinated hydrocarbons, such as DDT, have been found to resist breakdown and have accumulated at high levels in fields receiving frequent application. In the search for other more readily degraded compounds exhibiting insecticidal activity, attention has been drawn to the organophosphates. The toxicity of this group of compounds was apparently extensively studied in Germany during the 1940's. The pesticide activity of some organophosphates was reported subsequently by G. Schrader (as referred to in 17). A number of insecticidal organophosphate compounds are now commonly marketed. The general formula for these compounds

is $(RO)_2P(S \text{ or } O)OX$, where R is usually an alkyl or alkoxy compound and X appears to be a carrier or stabilizer, ranging from a substituted pyrimidine, nitrated phenol, to an ethylmercaptoethyl group. One such compound, used as a general insecticide, is O,O-diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorothioate, with the trade name of Diazinon, manufactured by the Geigy Chemical Corporation. Diazinon has been on the market for about 12 years and has been successfully used as an insecticide. It has been reported to have been metabolized by plants. Following one spraying of Diazinon, 50% of the original amount (determined by anticholinesterase activity) was detected after 25 days; at 63 days, 20% was detected (6). Ring-labeled $^{14}C$-Diazinon was reported to have been broken down and metabolized by tomatoes, as detected by the evolution of $^{14}CO_2$ and the isolation of radioactive 2-isopropyl-4-methylpyrimidinyl-6-ol, and by spinach and beans forming diazoxon (oxygen analog of Diazinon) (62). In another study, side chain labeled $^{14}C$-Diazinon was found to be rapidly translocated in bean plants from the point of application on leaves to root tips in unaltered form. No radioactive metabolites were found (34).

Evidence is available for insect metabolism of Diazinon. Obviously, if the lethal activity of Diazinon in insects is phosphorylation of a cholinesterase, then the removal of the phosphorus side chain would be the true

detoxication mechanism (49). Detoxication is in evidence in insects that have developed resistance to Diazinon. Dephosphorylation was found to occur with dimethyl, diethyl, but not diisopropyl, Diazinon (58). In a study of resistant (R) and susceptible (S) cockroaches and houseflies, 4 hours following Diazinon application, the S strains had 2-1/2 times the amount of diazoxon as the R strains, suggesting that differences in the diazoxon level between R and S are responsible for the selectivity of the S strains. It was also concluded that the R strains could remove Diazinon faster than the S strains (42). A phosphatase from R houseflies was found to be able to degrade diazoxon but not Diazinon (58). It had been reported previously that aliesterase activity in R strains was lower than in S strains (75), which led to the conclusion that perhaps the enzyme (the phosphatase referred to above) responsible for hydrolysis of the oxygen analog of Diazinon was made at the expense of regular aliesterases found in S strains, through a single gene mutation converting part of the aliesterase into a phosphatase (76).

The metabolism of Diazinon has been reported in mammals. When $^{32}$P-Diazinon was fed to a cow, clearing of radioactivity from blood, milk, and feces occurred in 96 hours. Within 36 hours, 74% of the radioactivity had been accounted for in the urine. At 36 hours, of the radioactivity found, 0.2% was Diazinon, 50% was 0,0-diethyl-phosphorothioc acid,

or Diazinon side chain, and 45% was 0,0-diethyl phosphoric acid (64). The diethyl phosphorothioic acid was formed by direct hydrolysis of the parent molecule; the diethyl phosphate was derived from oxidation of the parent molecule and subsequent hydrolysis and/or from the oxidation of the diethyl phosphorothioate (64). It may have proved more valuable if some portion of the ring moiety had been labeled, for nothing was mentioned as to the fate of the pyrimidinyl portion of the molecule. The same observation holds true for another report involving the feeding of $^{32}$P-Diazinon to rats. Alkyl phosphate hydrolysis was reported; this hydrolysis was proportionately greater with dimethyl than with diethyl phosphorothioate. Also noted was that the oxygen analog of the side chain was more readily hydrolyzed than the phosphorothioate (59). In this study, 24% of the $P^{32}$ was recovered as diethyl phosphate; 71% was found to be in the side chain hydrolyzed from the parent molecule. In cockroaches, also used in this work, the levels were 41% oxidized to phosphate and 59% phosphorothioate side chain (59).

As with other insecticides, Diazinon exerts influence on its environment. An application of 300 ppm of Diazinon stunted the growth and development of bean plants (71). When used in dry sugar bait at 0.1% concentration, Diazinon gave a 99% kill of houseflies, resistant to DDT, in 16 hours (32). The acute oral $LD_{50}$ for rats is 100 to

150 mg Diazinon/kg. Cholinesterase in plasma, red blood cells, and brain tissue is inactivated by $3.79 \times 10^{-4}$, $3.79 \times 10^{-5}$, $3.79 \times 10^{-6}$ M Diazinon respectively; larger doses resulted in no gross signs of toxicity, but did retard cholinesterase activity (14).

Few references are found that refer to Diazinon breakdown in the soil or by soil isolates. In one of the more recent works (33), it was found that when an Arthrobacter sp. and a Streptomyces sp. were incubated together in a medium containing ring labeled $^{14}$C-Diazinon, radioactive $^{14}CO_2$ was recovered, as $Ba^{14}CO_2$. Autoradiographic evidence indicated the appearance of two other metabolites of ring labeled Diazinon not found on $^{14}$C-Diazinon incubation with either culture individually. In another study of Diazinon metabolism, it was reported that rumen microorganisms only slightly reduced the level of Diazinon, indicating an inability of the rumen microflora to metabolize Diazinon (21).

In a study of the effect of insecticide on soil microorganisms, organophosphates had no effect on fungal or Streptomyces count. In field studies at an application rate of 10 lbs. per acre, Diazinon increased bacterial numbers; in vitro applications from 40 to 1500 ppm caused a similar increase. $CO_2$ evolution also rose, seemingly proportional with the level of Diazinon applied (42). In another report, contrary results were noted. Organophos-

phates were found to cause an initial increase and subsequent decrease in $CO_2$ production by the soil microflora, the degree of effect related to the concentrations of organophosphates applied (8). It is clear, therefore, that further investigation is necessary to reconcile such inconsistencies.

Much fundamental work has been reported on the chemistry of the Diazinon molecule. It has been established that one of the major hydrolysis reactions of Diazinon is the removal of the side chain (15,35,59,64,76), followed by or in place of oxidation of this side chain (56,59,64). The presence of diazoxon has been frequently reported in Diazinon studies (17,35,42,49,62,74,76). The only reference to the detection of a pyrimidine residue of Diazinon metabolism identified the substance as 2-isopropyl-4-methylpyrimidin-6-ol, a substance used in the synthesis of Diazinon (62); this occurred in tomato plants treated with Diazinon. No other breakdown products were found. Utilization of the ethyl esters as the source of carbon has been reported for $^{14}C$-ethyl ester Diazinon (34); however, it was not determined whether the ethyl ester was removed from the phosphorus prior to or subsequent to hydrolysis of the side chain.

In an effort to conceptualize more clearly the points of attack on the Diazinon molecule and to understand why these particular points were susceptible to attack, a further study was conducted in the literature available on

Diazinon-like substances. For example, it has been reported that the Diazinon molecule was dephosphorylated most readily if the dialkyl substitutions on the phosphorus were methyl groups instead of ethyl groups. When the dialkyl groups were isopropyl, no dephosphorylation occurred (77). It is stated that the above phenomenon occurs because as the alkyl groups increase in carbon atoms, the degree of electron release from the alkyl group increases, lowering the reactivity of the phosphorus atom with esterase (15). It has been suggested that the electronic effects on the susceptibility of the molecule to hydrolysis may be less important than the influence of steric effects. Thus, a branched alkyl group or very large X group could sterically hinder the hydrolytic attack (55).

In another report, a correlation between insect cholinesterase inhibition and the lability of the P-O-X bond was found to be excellent, but, again, mention was also made of the influencing factor of steric hindrance (31). The stability of the molecule could be further enhanced if the R group were alkoxy or, better still, an alkylamide (17). In a study of insect resistance to Diazinon, it was found that the level of diazoxon in insects determined sensitivity to the insecticide (42). In toxicity studies of Diazinon, it was found that Diazinon had a mouse toxicity of 120 mg/kg per oral dose, and a pseudocholinesterase $I_{50}$ of 0.8 mg%, whereas the diazoxon mouse toxicity was 11 mg/kg and pseudo-

cholinesterase $I_{50}$ of 0.00018 mg% (45). In comparing the rates of hydrolysis at pH 7 in one report, diazoxon was hydrolyzed at 10 times the rate of Diazinon (35); the difference in hydrolysis rates was reported elsewhere to be between 10 and 100 times; phosphorothioate esters were 1000 times less reactive with cholinesterase than were phosphate esters (49).

In consideration of the greater stability of the phosphorothioate than the phosphate, it is worthwhile to examine the electronegativity values of phosphorus, sulfur, and oxygen, which are 2.1, 2.5, and 3.5, respectively (49). From such observations, it would appear that there would be less stress in the molecule if the electronegativity values were closer, as with phosphorus and sulfur (2.1 to 2.5), as opposed to phosphorus and oxygen (2.1 to 3.5). With greater stress arising from uneven electron distribution, the molecule naturally would be more susceptible to hydrolysis and enzymatic attack. Then, too, the greater anticholinesterase activity of the P=O molecule could be explained, for it would be reasonable to suggest that when the phosphorus atom becomes more positive, or a better electrophilic reagent, anticholinesterase activity would increase (55). The organophosphate insecticides act as anticholinesterases. In this process, one of inhibition, the enzyme, cholinesterase, becomes phosphorylated at its esteractic site in a more or less irreversible chemical reaction (29), in which

the phosphorus makes an electrophilic attack on the active site. For a more detailed study of the phosphorus-enzyme reaction, the reader is referred to O'Brien's fine volume on Toxic Phosphorus Esters (55), and a brief review of the use of phosphates in elucidation of the structure of active sites in esterases (19,30).

Innate to the phosphorothioate molecule $(RO)_2P(S)OX$ is the phenomenon of isomerization at elevated temperatures or under ultraviolet light (17). The first report of isomerization was in 1911, when a monothiophosphate, P(S)OR, was heated in a sealed tube for 3 to 4 hours at 100°C; at the end of the test, 50% of the original pure compound was recovered as the S-alkyl isomer P(O)SR (26). Similar isomerization has been reported for Parathion, methyl parathion, and Malathion; the "heat isomerides" possessed a greater anticholinesterase activity than the parent compounds, the amount of inhibition being a measure of the degree of isomerization (5,50). A second type of isomerization has been reported in which $(RO)_2P(S)OX$ was transformed to $(RO)_2P(O)SX$. This reaction was more common than the first described, when X was other than an aryl group (55). One study, in which intermediates were formed, detailed the isomerization of Systox (B-ethylmercaptoethyl-diethyl-thionophosphate) (5). Generally, isomerization of phosphorothioate increases the inhibition of cholinesterase and the vulnerability of the compound to hydrolysis (48,55).

Seemingly, most investigators have been satisfied with establishing a hydrolysis of the Diazinon molecule and perhaps the oxidation of the phosphorothioate to phosphate. Little effort has been put into determining the fate of these hydrolysis products. One of the products, more familiar to workers, is the pyrimidinyl residue. A variety of pyrimidinyl residues were obtained when Diazinon and small amounts of $H_2O$ were heated together. Of the initial 100 g of Diazinon heated, 53.6 g were recovered as pyrimidinyl residues. The residues included Diazinon, 20.5 g; 2-isopropyl-4-methyl-6-hydroxy pyrimidine, 0.5 g; 2-isopropyl-4-methyl-6-ethoxypyrimidine, 8.2 g; 2-isopropyl-4-methyl-6-ethylmercaptopyrimidine, 4.3 g; and bis-[2-isopropyl-4-methyl-pyrimidyl-(6)]-sulfide, 20.1 g (45). None of these pyrimidinyl residues had high mammalian toxicity or high anticholinesterase activity. Extremely high toxic products of this "artificially aged" Diazinon were found to be monothio-TEPP with a cholinesterase $I_{50}$ of 0.000055 mg% and dithio-TEPP cholinesterase, $I_{50}$ = 0.00079 mg%. Tomato plants were reported to form 2-isopropyl-4-methyl-pyrimidinyl-6-ol from Diazinon (62). Such pyrimidinyl residues are unlikely to be resistant to degradation. Metabolism of normally occurring pyrimidine has been reported (37) and reviewed elsewhere (1,7,13,60). Pyrimidines with alkyl substitution in positions 2, 4, or 6 would become "active" by an electronic effect. This electronic effect is brought

about by the electron-attracting properties of the 2 nitrogen atoms (1,7,13), thus making alkyl groups in position 2, 4, or 6 electron-poor and, therefore, susceptible to a nucleophilic attack.

In a study of pyrimidine metabolism by a gram-positive non-spore-forming rod isolated from the soil, it was found that when position 6 of the heterocyclic ring was substituted, oxidation was very slow, unless the substitution was a hydroxyl group (79). Also noted was the importance of the double bond between positions 5 and 6. Little or no oxidation was detected if the compound was saturated. From the above information, it becomes clear as to the manner in which the pyrimidinyl residue of Diazinon can be attacked. The isopropyl and methyl group at position 2 and 4, respectively, are "active" and susceptible to nucleophilic attack. At position 6 is a hydroxyl group and the ring is unsaturated. Following oxidation of the 6-hydroxyl group, it seems possible that the ring may be opened. There has been evidence for degradation of the pyrimidinyl ring; when Diazinon, labeled in the ring, was placed in a culture with an Arthrobacter sp. and a Streptomycetes sp., $^{14}CO_2$ was evolved (33). There is, then, some knowledge of the metabolism of the pyrimidinyl residue of Diazinon. However, no work has been reported on the metabolism of the side chain alone. The purpose of the work reported here, therefore, was to clarify the soil microfloral response to the presence of

Diazinon and to study the breakdown of the side chain by soil microorganisms.

# METHODS AND MATERIALS

I. The response of the soil microflora to the presence of Diazinon

A. Population studies. Soil was obtained from the 0 to 6" level of a densely-wooded area, a corn field, a dried swamp, and from the bottom of a pond, respectively. A 1 g sample of each of these soils was put into sterile dilution bottles; the samples were diluted to $2 \times 10^{-1}$, $10^{-1}$, $2 \times 10^{-2}$, $10^{-2}$, $2 \times 10^{-3}$, $10^{-3}$, $2 \times 10^{-4}$, and $10^{-4}$ concentrations. One ml of each sample was inoculated into 50 ml of modified Morris medium* in a 250-ml Erlenmeyer flask containing, as the experiment required: 1 ml 50% glucose, 1 ml of 10% carbohydrate free yeast medium, or 0.5 ml Diazinon (1:0.5 in ethanol). The flasks were placed on a rotary shaker at about 30°C. After 3-days incubation, flasks were examined for growth. Aliquots were removed from where Diazinon served as carbon source and growth occurred, and these were streaked onto plates of nutrient agar or soil extract agar*; control flasks corresponding to the Diazinon-containing ones, but with glucose, were also streaked. After 5 days, the plates were examined for growth. Colonies from Diazinon cultures, differing in morphology from those obtained in glucose-containing cul-

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*The medium constituents are listed in the Appendix.

tures, were subcultured and maintained for further examination.

A study of soil microfloral response to Diazinon application was conducted under greenhouse conditions, using wooden boxes that contained a sandy loam soil mixture with a sod covering of Merion Blue Grass (Poa pratensis, L. var.). Diazinon was applied at the recommended rate of 7 ounces per thousand square feet and at double that rate at one-month intervals for 3 months. Five months after the final application, soil core samples were removed from each box, and 1 g of each sample was used in the population study. Control boxes received no Diazinon treatment. Soil sample dilutions to $10^{-4}$, $10^{-5}$, and $10^{-6}$ were made in sterile water. Pour plates were made, using 1-ml aliquots from each dilution on nutrient agar and soil extract agar. Five pour plates were made in both media for each dilution; the agar was kept in its liquid form at 45°C in a water bath prior to pouring. After 4 days, the plates were counted. Again, colonies found on plates from Diazinon-treated soils, not similarly present on plates from control soils, were subcultured to be used in subsequent investigations.

B. Diazinon sulfur utilization by selected isolates. It has been reported that the first point of attack on Diazinon by soil microorganisms was the sulfur atom of the Diazinon molecule (34). The ability of the soil isolates to use Diazinon as a sulfur source in a sulfur-free minimal

salts medium was therefore studied. It was intended, thereby, to eliminate all but those isolates that could most readily attack the Diazinon molecule at its purportedly weakest point. Three different media were used: 1) sulfur-free minimal salts medium, also known as modified Morris medium, and glucose; 2) sulfur-free minimal salts medium, glucose, Diazinon; and 3) minimal salts medium with sulfur and glucose. Isolates were grown in a peptone-glucose medium, harvested after 2 days, washed, and a heavy inoculum was made in the test medium. The flasks used were 250-ml nephloflasks from Bellco Glass, Inc., Vineland, New Jersey, with side arms capable of fitting into a Klett-Summerson Photoelectric Colorimeter. Readings were taken through a red filter at 670 mμ. Ten ml of medium was used in each flask. Readings were taken daily for 4 days.

II. Metabolism of the diethyl phosphorothioate moiety of the Diazinon molecule

A. Warburg respirometric studies. Respirometric studies were conducted on the capability of isolates to metabolize the Diazinon side chain, O,O-diethyl-phosphorothioate (potassium salt) obtained from the American Cyanamid Co. Since Diazinon is only slightly soluble in water, it was solubilized in ethanol prior to its introduction into various growth media. The ability of several of the isolates to grow in simple or supplemented medium with

ethanol as carbon source was therefore investigated. In a preliminary study, two isolates were inoculated into the 4 following types of media: 1) 25 ml minimal salts medium with 0.5 ml 50% glucose; 2) 25 ml minimal salts medium and 1.0 ml 98% ethanol; 3) 25 ml minimal salts medium, 1.0 ml ethanol, and 0.05% yeast extract; and 4) 25 ml minimal salts medium, 1.0 ml ethanol, and 0.01% yeast extract. In the second and last test of this series, three other isolates were inoculated into the same 4 media described above, and two others: 5) 25 ml minimal salts medium and 0.05% yeast extract and 6) 25 ml minimal salts medium and 0.01% yeast extract. Growth in both cases was read daily on a Klett-Summerson Photoelectric Colorimeter.

The isolates that utilized the sulfur atom of the intact Diazinon molecule were used in the following studies. They were grown in various media: peptone glucose, yeast-extract and glucose, or yeast-extract and ethanol. The medium was dispensed as either 100 ml in a 250 ml Erlenmeyer or 700 ml in a 2800 ml Fernbach flask. Subsequent to harvesting and washing in sterile phosphate buffer, the cells usually were starved to decrease endogenous respiration levels. The respirometric studies in the Warburg apparatus followed the method of Umbreit, Burris, and Stauffer (73). In the first test, the substrate(s) to be utilized (glucose 0.5% and/or side chain 0.5%) were placed in the center vessel and the isolates in the first side

arm. Following equilibration, the side arms were tipped in and the isolates came in contact with the substrate(s). In succeeding tests, isolates were placed in the center vessel and substrate(s) (glucose 0.005% and/or side chain 0.1%) were placed in the side arms. After the reaction vessels were equilibrated, the first side arms were tipped; then, as $O_2$ uptake reached a plateau, contents of the second side arms were introduced. During this second series of experiments, various concentrations of side chain were used (0.01%, 0.1%, 0.5%, 1.0%, and 5.0%) to study the effect of concentration on $O_2$ uptake. These tests were sustained for 5 to 6 hours. In the third series, both $CO_2$ evolution and $O_2$ uptake were studied and an attempt was made to relate the $CO_2/O_2$ ratio to the type of metabolic reaction involving the side chain.

B. Detection of metabolites in the degradation of Diazinon side chain. Thin-layer chromatography was used in attempts to identify metabolites of Diazinon side chain with solvent systems including: chloroform-methanol, 9:1, 5:5, 0:1, n-butanol-pyridine-water 6:4:3, methanol-methylene chloride-ammonia (10%) 20:80:3 (69), n-hexane-water 8:2, and n-butanol-acetic acid-water 12:3:5, 60:15:25.

Thin-layer plates were prepared from Adsorbosil-1, from Applied Science Laboratories, State College, Pa., at a 0.250-mm thickness and activated for 1/2 hour at 100°C; material was applied with a blunt-tipped 10-µl syringe. When the solvent front was about 10 mm from the top of the

plate, the plate was removed from the solvent tank and air-dried. Detection of the phosphorothioate group was achieved by spraying* with a solution of $(NH_4)_2PdCl_4$ after (3) or 2,6-dibromo-N-chloro-p-quinoneimine (DBCQ) after (10) modified from P=S detection on a paper chromatogram (47). Both sprays were specific in their reaction with the P=S group. A positive result with the palladium spray was indicated by the appearance of a yellow color; for the DBCQ spray, a positive area was red. To detect phosphate esters, the following sprays were tried: Dittmer's phosphate spray (25), Hanes-Isherwood's phosphate spray (36), modified after (23), and a method of phosphate detection involving ferric ions and salicylsulfonic acid (78).

In order to economize on the quantity of adsorbosil used in screening these systems, microscope slides were coated with 2 ml of the adsorbosil slurry. These slides were then spotted with 10 μg of the side chain, placed in 300-ml beakers containing 10 ml of the solvent systems tested; the solvent front was permitted to run up to within 1/4" of the leading edge of the slide. Following spraying with ammonium palladium chloride, the solvent was chosen that gave an $R_f$ of 0.4 to 0.5 and kept the side chain in a small spot as it migrated up the slide.

The quantitation of the concentration of the phosphoro-

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*Spray compositions given in Appendix.

thioate-containing material followed the method of Akerfeldt and Lovgren (3). A standard curve was made by preparing 0.1-ml samples of a series of solutions containing from 1 μ mole/ml to 10 μ mole/ml of the side chain and adding 0.5% of the reagent (20 μ moles/ml of $(NH_4)_2PdCl_4$ in 1 N HCl) followed by 2.4 ml of 1 N HCl. Optical density was read at 400 mμ. To determine P=S concentration in test solutions, a dilution was made, so that the concentration of side chain would be between 1 and 10 μg/ml; a 0.1-ml sample of the appropriate dilution was then assayed in the same manner as the standard solutions.

# RESULTS

## I. The response of the soil microflora to the presence of Diazinon

A. Population studies. To isolate organisms whose presence was stimulated through the action of Diazinon in the soil, inoculations from forest and field soil, diluted in sterile water blanks to levels of $2 \times 10^{-3}$ and $2 \times 10^{-4}$, were made into 250-ml Erlenmeyer flasks containing minimal salts medium. The only growth detected after 4 days incubation was in the flasks containing glucose as the carbon source; no growth was seen in the Diazinon-containing flasks.

It was conjectured that perhaps the pH of the Diazinon medium may have been altered to such an extent that microorganisms could not develop. To investigate this possibility, a further test was made in which samples of field and forest soils were diluted to $10^{-3}$ and $10^{-4}$ concentrations and inoculated into the media previously described. Once again, growth occurred only in flasks in which glucose served as carbon source; the pH of the flasks supporting growth ranged from 6.0 to 6.5, and the pH of the flasks supporting no growth ran from 6.7 to 7.1.

Other soils were also examined. One-ml samples from $10^{-2}$ and $10^{-3}$ dilutions of swamp and pond soil were inoculated into the same type of medium. On the succeeding day,

growth was observed in all flasks. Loops of medium from all the flasks were streaked out on nutrient agar and soil extract agar. No significant change in the microbial population was detected by this method; nevertheless, a few colonies were selected which occurred on test but not on control plates.

Soils that actually had received a field level application of Diazinon to control insects were tested, in which Diazinon would have been in contact with the soil for 90 days. Plots in the football field at the University stadium had received Diazinon applications varying in concentrations: 0; 1/2 the recommended level, 7 ounces/1000 square feet (manufacturer's recommendation); 14 ounces/1000 square feet; or 28 ounces/1000 square feet, in May, 1966. Core samples from the 0 to 6" level were removed in September, 1966, from the centers of the plots so treated. Dilutions made of 1 g portions of the core samples were subsequently pour-plated with nutrient agar and soil extract agar. Plates were counted 8 days following inoculation. The results of this test are given in Table 1. Although a decrease in bacterial numbers was indicated by the data obtained, reduction of the bacterial population noted was not statistically significant by the Student-T test.

It was decided to make a second study of the effects of Diazinon on soil microflora. This survey was conducted

TABLE 1. Influence of Diazinon on numbers of soil microflora in field soil under turf.

| Diazinon Concentration | Plating Medium | Microbial Population x $10^5$/g | | |
|---|---|---|---|---|
| | | Bacteria | Fungi | Actinomycetes |
| Oz/1000 sq. ft. | | | | |
| None | Nutrient Agar | 58 | 4 | 2 |
| 3.5 | | 42 | 0 | 2 |
| 7.0 | | 24 | 2 | 6 |
| 14.0 | | 26 | 2 | 3 |
| 28.0 | | 30 | 1 | 1 |
| None | Soil Extract Agar | 44 | 2 | 3 |
| 3.5 | | 70 | 2 | 2 |
| 7.0 | | 35 | 2 | 4 |
| 14.0 | | 34 | 2 | 2 |
| 28.0 | | 15 | 2 | 1 |

under greenhouse conditions; the soil studied was a sandy loam mixture with a moisture-holding capacity of 48% and a sod covering of Merion Kentucky Bluegrass. Diazinon was applied at monthly intervals for 3 months at the recommended rate of 7 ounces/1000 square feet and at twice the recommended rate. Control boxes received no treatment. Five months after the final application, core samples were removed from the boxes; 1 g portions of each sample were diluted aseptically and pour plates of dilutions $10^{-4}$, $10^{-5}$, and $10^{-6}$ were made in nutrient agar and soil extract agar. At each dilution, five plates were poured for both media. The media were kept in liquid form in a constant temperature water bath at 45°C. The plates were counted 4 days after pouring. Results of the test may be seen in Table 2. Only the count for the $10^{-5}$ dilution is reported, as counts at the lesser dilutions were over 300 per plate, and the greater dilution counts were generally less than 30. Following analysis of this data by a Student-T test, it was found that, in nutrient agar, the bacterial count of soil receiving twice the recommended application rate of Diazinon was statistically lower than that of the control soil (at 95% confidence limits). In soil extract agar, the decrease in bacterial counts of both soils receiving the recommended level or twice the recommended level of Diazinon was also statistically significant. In those instances where colonies occurred in the test plates that occurred much

TABLE 2. Influence of Diazinon on numbers of soil microflora in greenhouse sandy loam soil.

| Diazinon Concentration | Plating Medium | Microbial Population x $10^5$/g | | |
|---|---|---|---|---|
| | | Bacteria | Fungi | Actinomycetes |
| Oz/1000 sq. ft. | | | | |
| None | Nutrient Agar | 151 | 3 | 2 |
| 7.0 | | 71 | 3 | 3 |
| 14.0 | | 22* | 1 | 2 |
| None | Soil Extract Agar | 103 | 3 | 1 |
| 7.0 | | 36* | 3 | 2 |
| 14.0 | | 22* | 4 | 2 |

*Statistically significant at 95% confidence limits by Student-T test.

less frequently in the control plates, the colonies were subcultured and the microorganisms represented by these colonies were isolated. Sixteen isolates were obtained in this manner, representing organisms arising in response to the presence of Diazinon.

In a study of the utilization of the Diazinon molecule as the sole source of sulfur, phosphorus, nitrogen, or carbon by a soil isolate, it was suggested that the isolate could most readily remove the sulfur atom and assimilate it in a sulfur-free medium (34). Thus, Diazinon-sulfur utilization was the first criterion used to eliminate those organisms that could not readily attack Diazinon. Three isolates most readily utilized the sulfur: 4, 9, and 16; 3 others utilized Diazinon-sulfur somewhat less readily but were included in succeeding studies for purposes of comparison.

II. Metabolism of the diethyl phosphorothioate moiety of the Diazinon molecule

A. Warburg respirometric studies. One of the prime objectives of this study was to investigate the metabolism of the side chain moiety of the Diazinon molecule. Since the ethyl groups of the side chain provide the carbon to any organism growing on the side chain, several isolates were examined for their ability to metabolize ethanol as sole carbon source. The results of the first experiment are given in Table 3.

TABLE 3. Growth of soil isolates with ethanol as sole source of carbon.

| | Medium | Isolate No. | Turbidity-Klett Units Hours 0 | 28 | 168 | 212 |
|---|---|---|---|---|---|---|
| 1 | Minimal salts medium (MSM), glucose | 1 | 15 | 580 | 560 | 560 |
| | | 12 | 14 | 14 | 8 | 5 |
| 2 | MSM, ethanol | 12 | 15 | 13 | 4 | 4 |
| 3 | MSM, ethanol, 0.05% yeast extract | 1 | 15 | 52 | 450 | 550 |
| | | 12 | 13 | 62 | 50 | 52 |
| 4 | MSM, ethanol, 0.01% yeast extract | 1 | 18 | 30 | 425 | 440 |
| | | 12 | 14 | 21 | 19 | 20 |

Isolate no. 1 grew in minimal salts medium and could utilize ethanol as sole source of carbon, but no. 12 was not able to grow in the minimal salts medium even with glucose as carbon source. What growth did occur was in direct response to the amount of yeast-extract added. In the second and last experiment of this series, 3 other isolates were inoculated into 6 media previously described. The data are presented in Table 4. None of these isolates utilized ethanol as sole carbon source. Only no. 9 used glucose as a carbon source in minimal salts medium.

It was postulated that, in a respirometric study, indications of side chain metabolism would best be seen in a diauxie reaction in which glucose, in limiting concentrations, was the first substrate utilized in the reaction vessel, followed then by side chain utilization. In a preliminary study, isolates numbers 1, 9, and 12 were grown 48 hours in peptone glucose medium, harvested, washed three times in sterile phosphate buffer, and introduced into the Warburg vessels. Other reaction constituents were: glucose, 0.5%; side chain, 0.5%; KOH, 20%; buffer, 0.1 M phosphate at pH 7. Four reaction vessels with 0.2-ml KOH in the center well were assigned to each isolate. The contents of each series of vessels are indicated by the following:

TABLE 4. Further studies of the growth of soil isolates with ethanol as sole source of carbon.

| Medium | Turbidity-Klett Units Hours 0 | 10 | 24 | 33 | 58 | 75 |
|---|---|---|---|---|---|---|
| Isolate | | | | | | |
| No. 4 | | | | | | |
| 1 MSM, glucose | 1 | 14 | 92 | 28 | 24 | 22 |
| 2 MSM, ethanol | 2 | 15 | 18 | 15 | 19 | 16 |
| 3 MSM, ethanol 0.05% yeast extract (ye) | 3 | 25 | 46 | 49 | 42 | 46 |
| 4 MSM, ethanol 0.01% ye | 4 | 24 | 25 | 27 | 25 | 23 |
| 5 MSM, 0.05% ye | 2 | 24 | 80 | 60 | 68 | 75 |
| 6 MSM, 0.01% ye | 1 | 13 | 32 | 20 | 28 | 21 |
| No. 9 | | | | | | |
| 1 | 14 | 118 | 570 | 590 | 580 | 580 |
| 2 | 11 | 18 | 16 | 15 | 19 | 18 |
| 3 | 11 | 18 | 22 | 24 | 25 | 29 |
| 4 | 11 | 22 | 34 | 43 | 47 | 44 |
| 5 | 9 | 53 | 52 | 59 | 58 | 56 |
| 6 | 5 | 24 | 24 | 25 | 25 | 23 |
| No. 13 | | | | | | |
| 1 | 7 | 14 | 27 | 27 | 19 | 20 |
| 2 | 4 | 13 | 15 | 17 | 18 | 16 |
| 3 | 5 | 43 | 59 | 66 | 59 | 66 |
| 4 | 10 | 24 | 26 | 27 | 28 | 28 |
| 5 | 0 | 40 | 93 | 75 | 71 | 73 |
| 6 | 5 | 22 | 34 | 25 | 22 | 29 |

Center Vessel

| Buffer | Cells | Glucose | Side Chain |
|---|---|---|---|
| 1.0 ml | 1.0 ml | 1.0 ml | -- |
| 1.0 ml | 1.0 ml | -- | 1.0 ml |
| 2.0 ml | 1.0 ml | -- | -- |
| -- | 1.0 ml | 1.0 ml | 1.0 ml |

The results of this study are depicted in Figures 1 and 2. It will be noted that isolates nos. 1 and 9 were capable of metabolizing the Diazinon-side chain and isolate no. 12 failed to give any indication. It was apparent that the microorganisms used glucose preferentially. However, in the case of both isolates 1 and 9, side chain metabolism, in the presence of glucose, appeared enhanced. Endogenous $O_2$ uptake, for which the figures have been corrected, was also high, and this may have masked the metabolism of the side chain, since the cells had sufficient energy sources to preclude the need for exogenous substrate.

In a second Warburg study, substrates to be utilized were kept in the side arms of the reaction vessels until equilibrium had been obtained. Then the contents of the first side arm were poured into the center vessel and readings were taken. At a later interval, contents of the second side arm were introduced. The concentrations of solutions used were: 0.2-ml 20% KOH in center well of all reaction vessels; glucose, 0.005%; side chain, 0.1%, 0.1 M phosphate buffer at pH 7. Isolates nos. 4, 9, and 13 had

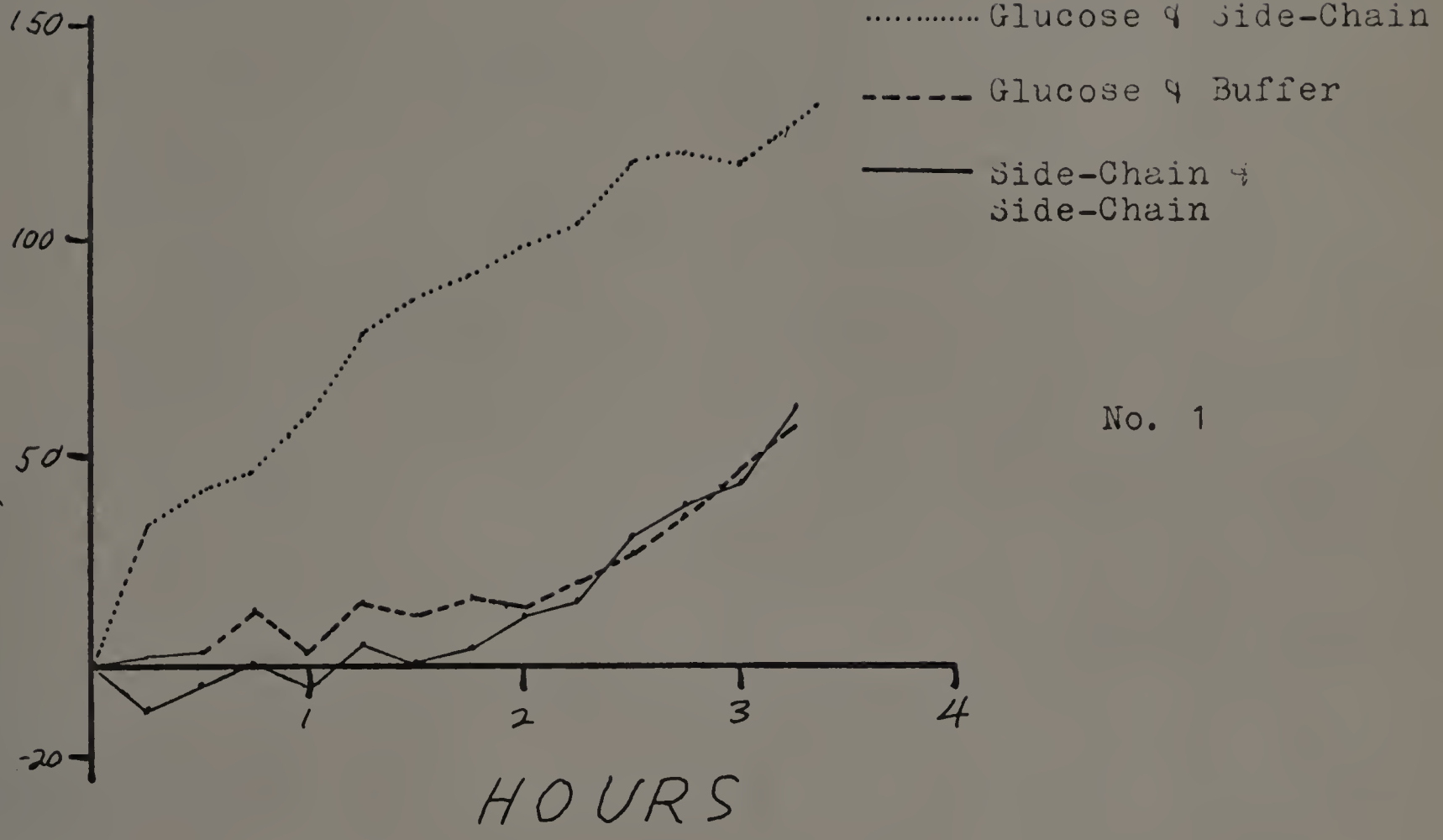



Figure 1
Metabolism of Diazinon side-chain by growing soil isolates.

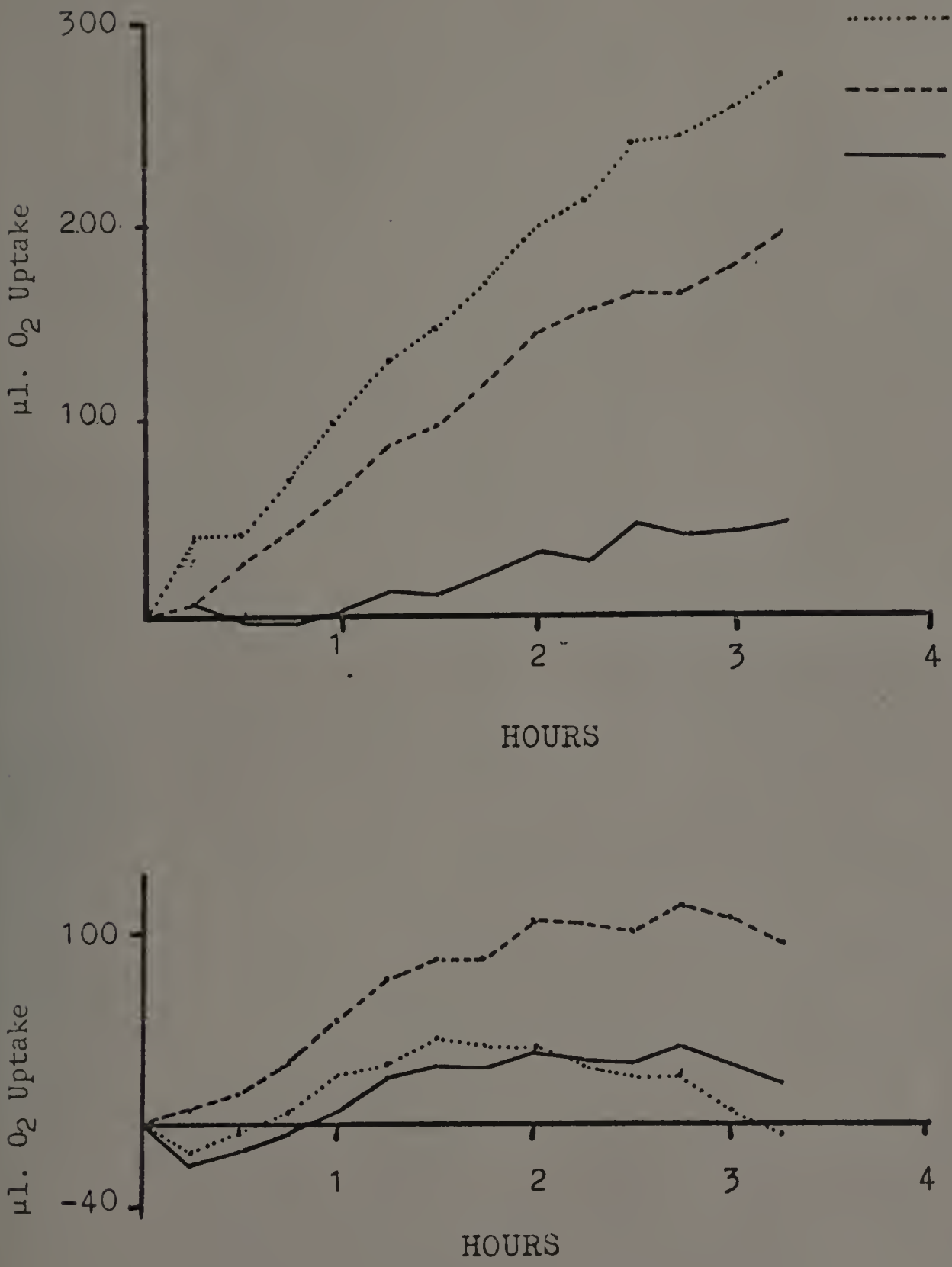



Figure 2
Metabolism of Diazinon side-chain by growing soil isolates.

been grown on a yeast extract glucose medium for 48 hours. The isolates were harvested, washed, and then starved in sterile buffer for approximately 40 hours. Following harvesting, they were resuspended in buffer prior to the experiment. Four vessels were assigned to each isolate, and each series was set up as follows: the KOH as already mentioned, and each vessel contained 1 ml of cells resuspended in buffer.

| Side Arm no. 1 | No. 2 |
|---|---|
| 1-ml buffer | -- |
| 1-ml glucose | -- |
| 1-ml glucose | 1-ml side chain |
| 1-ml side chain | 1-ml side chain |

Following equilibration, contents of the first side arm were poured into the center vessel. At four hours, contents of the second side arm were added. The experiment was terminated 90 minutes later. Results of this study are illustrated in Figures 3 and 4. It was observed that control flasks, without a compensating volume in the second side arm, were not adequate. Since one could not deduce whether the decrease and subsequent increase in $O_2$ uptake following the addition of side chain was due to metabolism of the added side chain or simply the mechanical resultant of the addition of liquid into the center vessel, the procedure for subsequent Warburg experiments was altered to

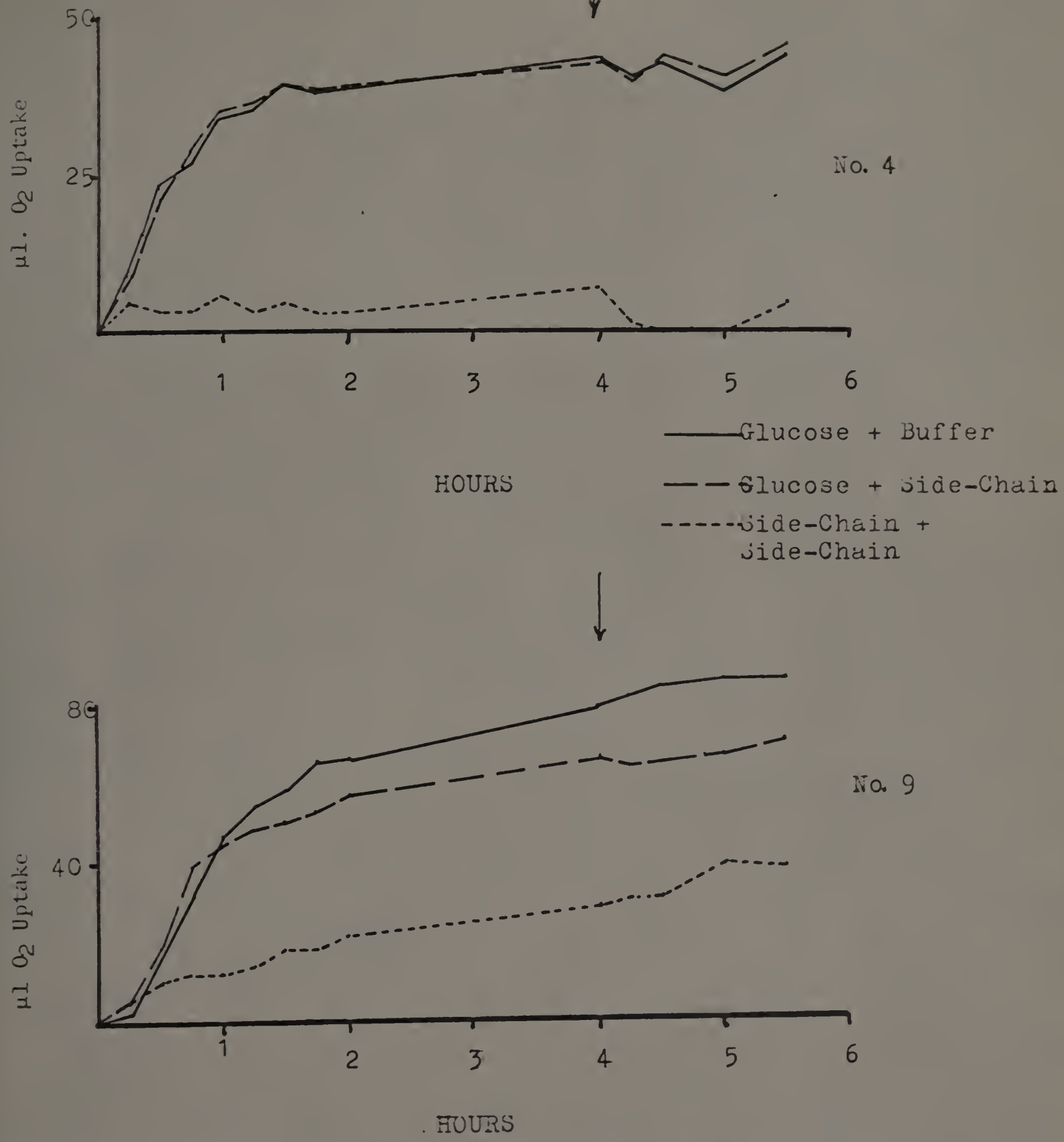



Figure 3
Metabolism of Diazinon side-chain by starved soil isolates.

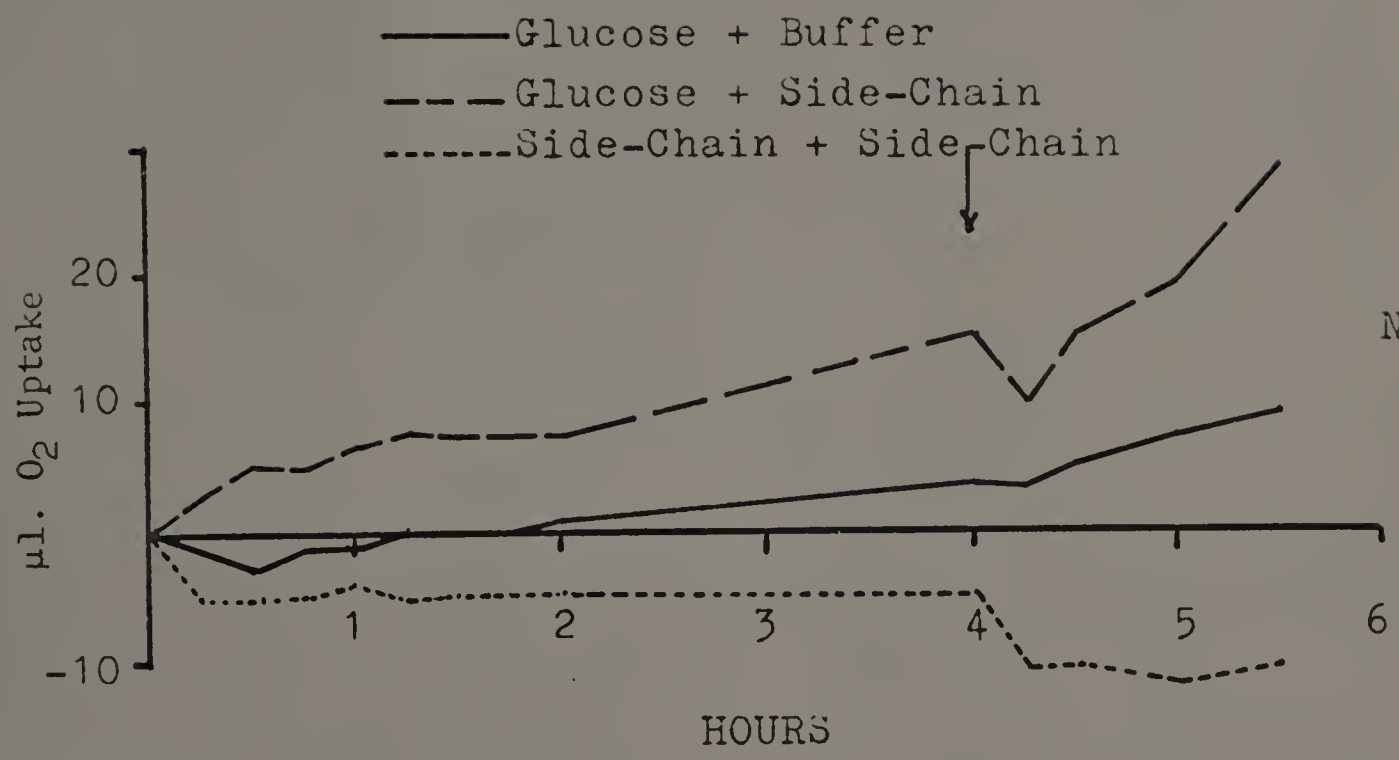



Figure 4
Metabolism of Diazinon side-chain by starved soil isolates.

include buffer in those side arms where no additional substrate was required.

Further studies were undertaken to confirm Diazinon-side chain utilization by demonstrating that $O_2$ uptake would reflect the concentration of side chain available. In a preliminary study, reducing the concentration of side chain resulted in reduced $O_2$ uptake for isolates nos. 9 and 16.

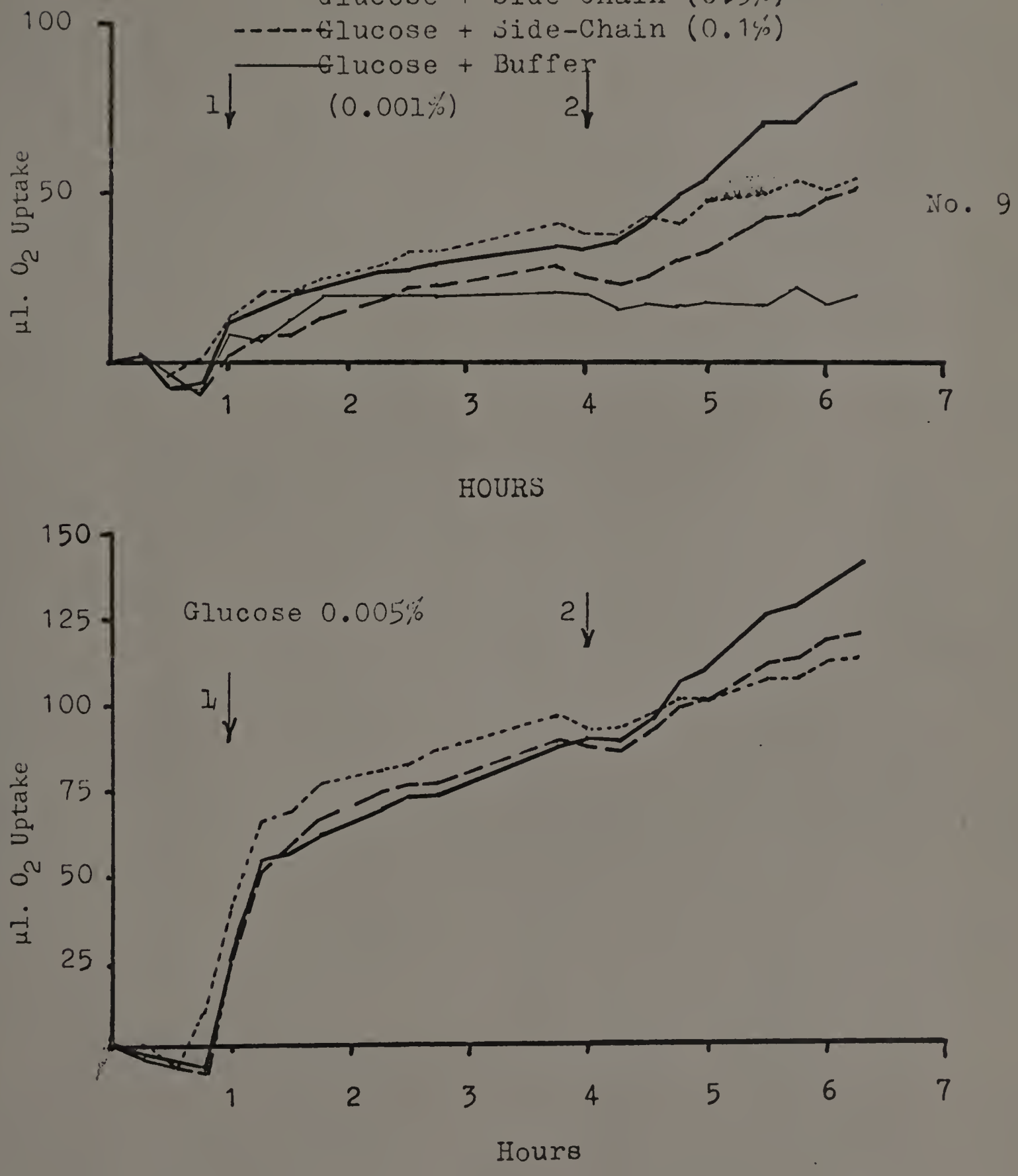
It was, therefore, decided to investigate further the influence of side chain concentration on $O_2$ uptake. Isolate no. 9 was grown for approximately 50 hours in minimal salts medium with glucose, harvested, washed, starved in phosphate buffer for 20 hours, harvested again, and resuspended in sterile buffer. The flasks were set up as indicated below. The center well of each vessel was filled with 0.2 ml of 20% KOH and each vessel had 1 ml of cells resuspended in buffer.

| Side Arm no. 1 | No. 2 |
|---|---|
| substrate 1 ml | substrate 1 ml |
| buffer | buffer |
| glucose-1 | buffer |
| glucose-1 | side chain-1 |
| glucose-1 | side chain-2 |
| glucose-1 | side chain-3 |
| glucose-2 | side chain-1 |
| glucose-2 | side chain-2 |
| glucose-2 | side chain-3 |
| glucose-3 | side chain-1 |
| glucose-3 | side chain-2 |
| glucose-3 | side chain-3 |
| glucose-3 | buffer |

Solution concentrations were: 0.001% glucose-1,

0.005% glucose-2, 0.1% glucose-3; 0.1% side chain-1, 0.5% side chain-2, 1.0% side chain-3. Endogenous readings were taken for one hour. The contents of the first side arm were poured into the center vessel then. Three hours later, the contents of the second side arm were added. The data obtained from this experiment are presented in Figures 5 and 6. It would appear that the concentration of glucose had little effect on the degree of the diauxie reaction obtained from the different levels of side chain concentration. However, the response to different levels of side chain was directly proportional to the side chain concentration. This would suggest that beyond the minimum threshold of glucose catabolism, the degree of Diazinon side chain metabolism reflects the specific capacities of the organism under test. What was observed was a differential response of the wide latitude of microorganisms inhabiting any soil micro-ecosystem.

The metabolism of isolate no. 16 was then studied in the same manner as isolate no. 9. The results are depicted in Figures 7 and 8. The response of isolate 16 to different levels of side chain concentration in general reflects the concentration of Diazinon side chain. The apparent discrepancy in the results in which a higher $O_2$ uptake was seen at 0.5% than at 1.0% may be attributed to technical error, since subsequent experiments indicated that this organism adhered consistently to a pattern of increased $O_2$




No. 9

Figure 5
Metabolism of Diazinon Side-Chain as a function of Side-Chain concentration.

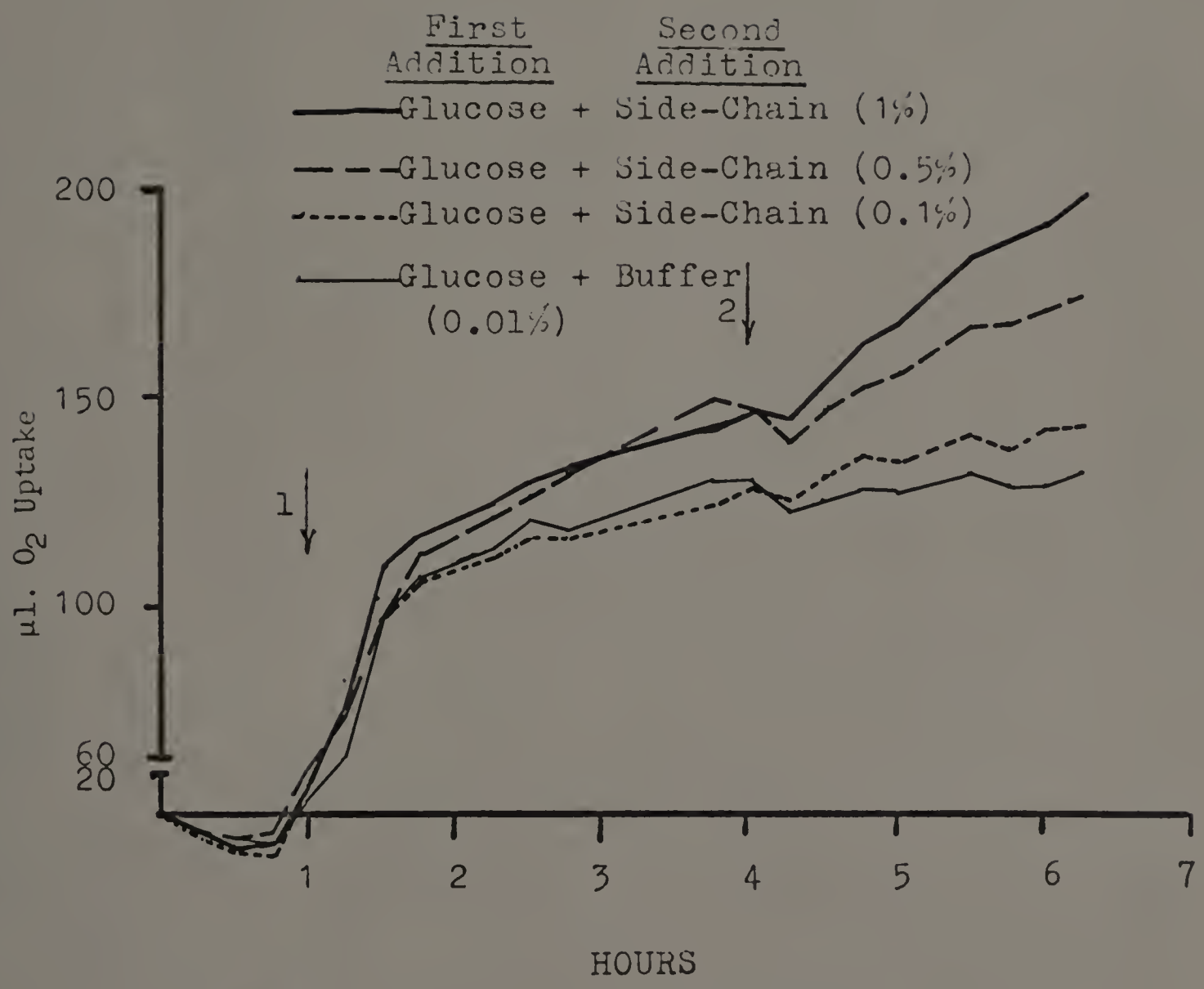
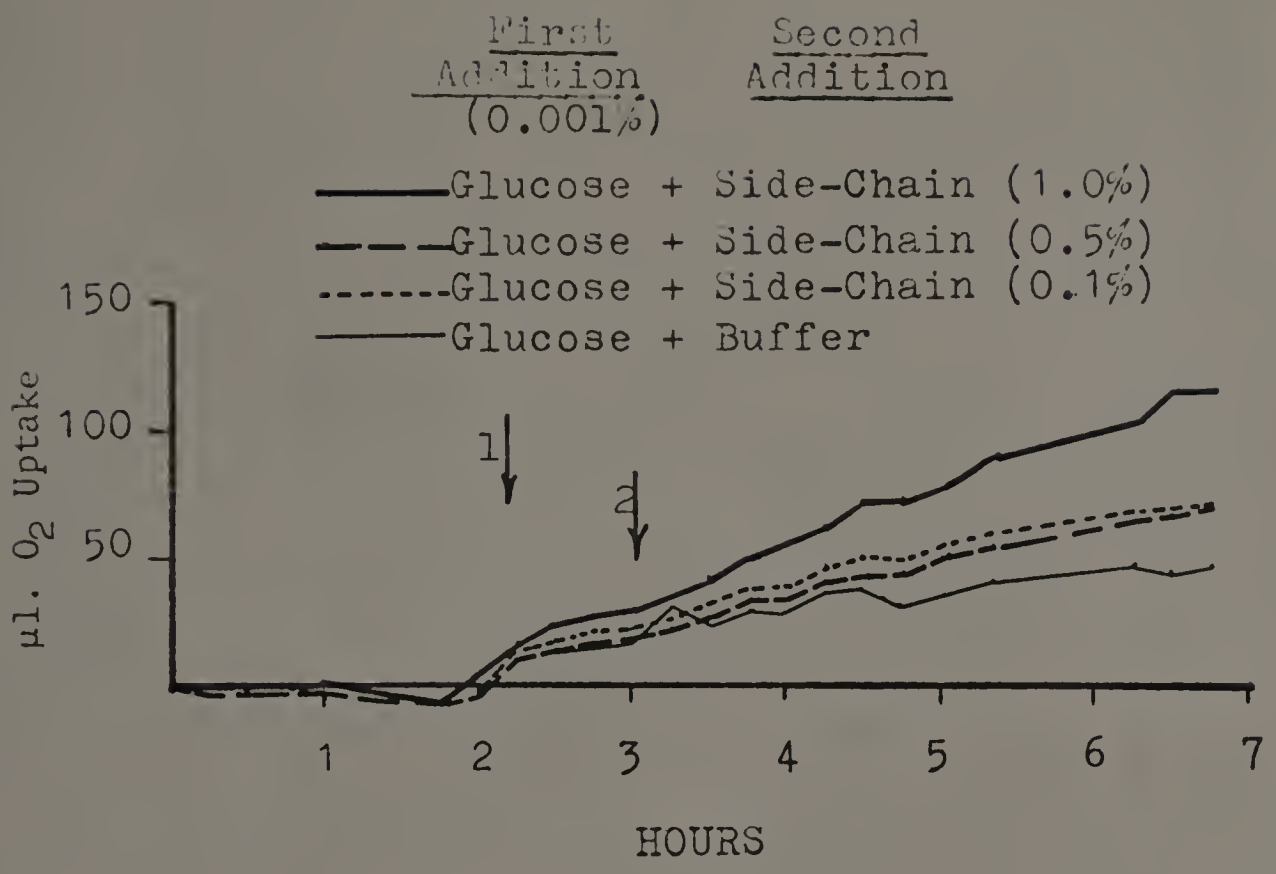



Figure 6

Metabolism of Diazinon side-chain as a function of side-chain concentration.




No. 16

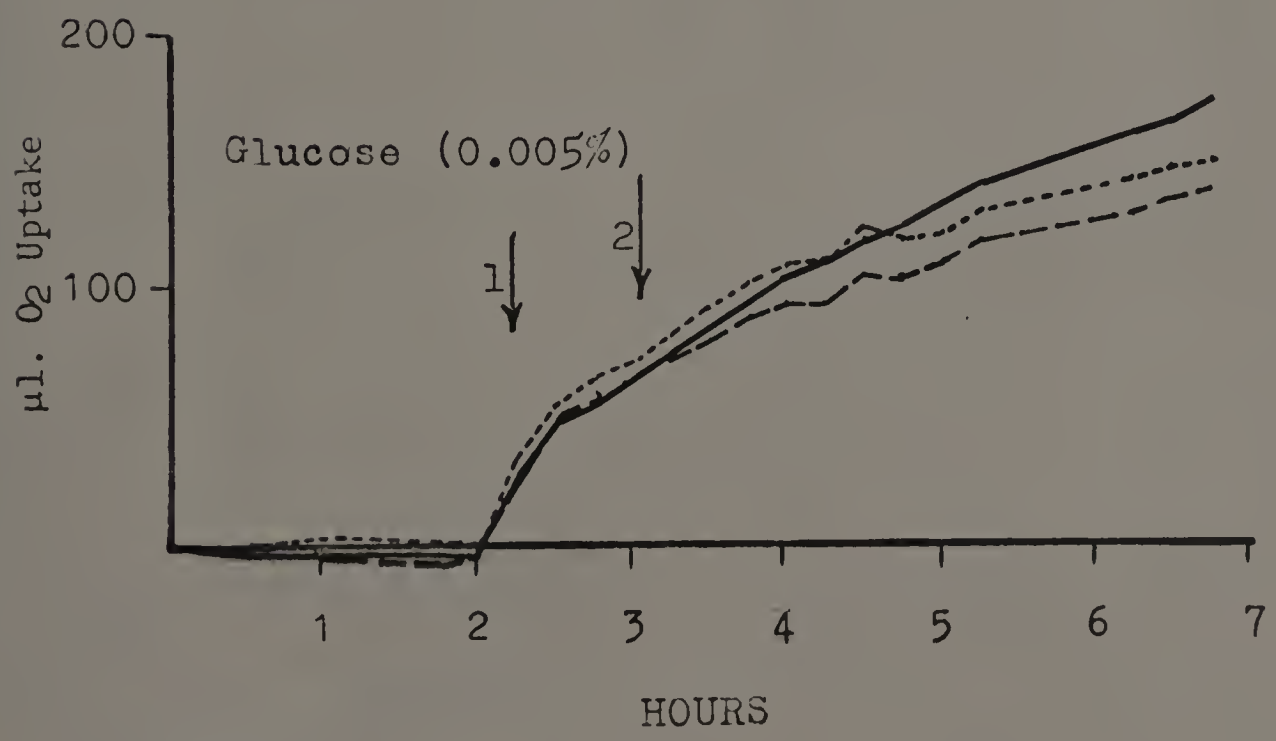
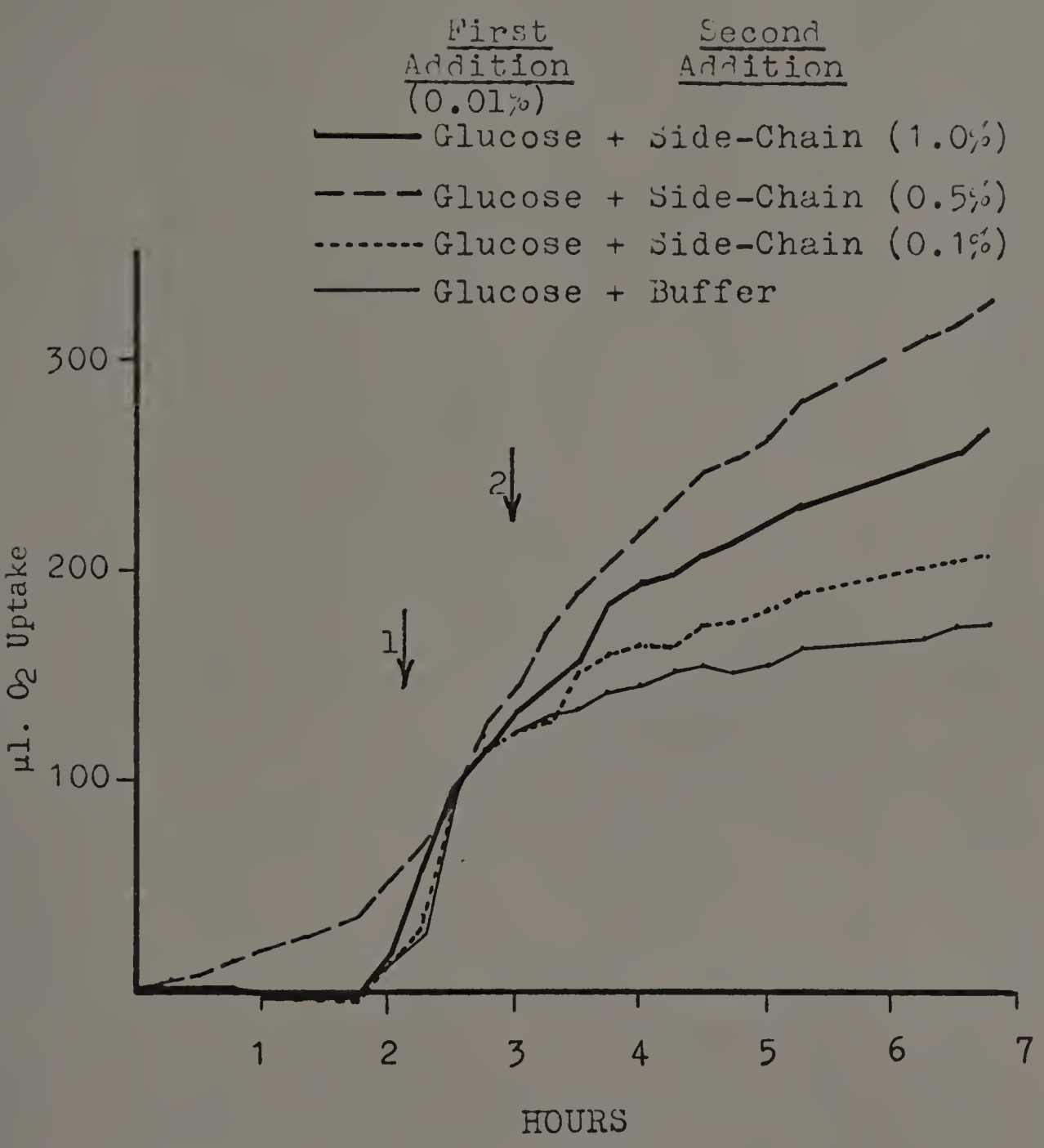



Figure 7.
Metabolism of Diazinon side-chain as a function of side-chain concentration.




No. 16

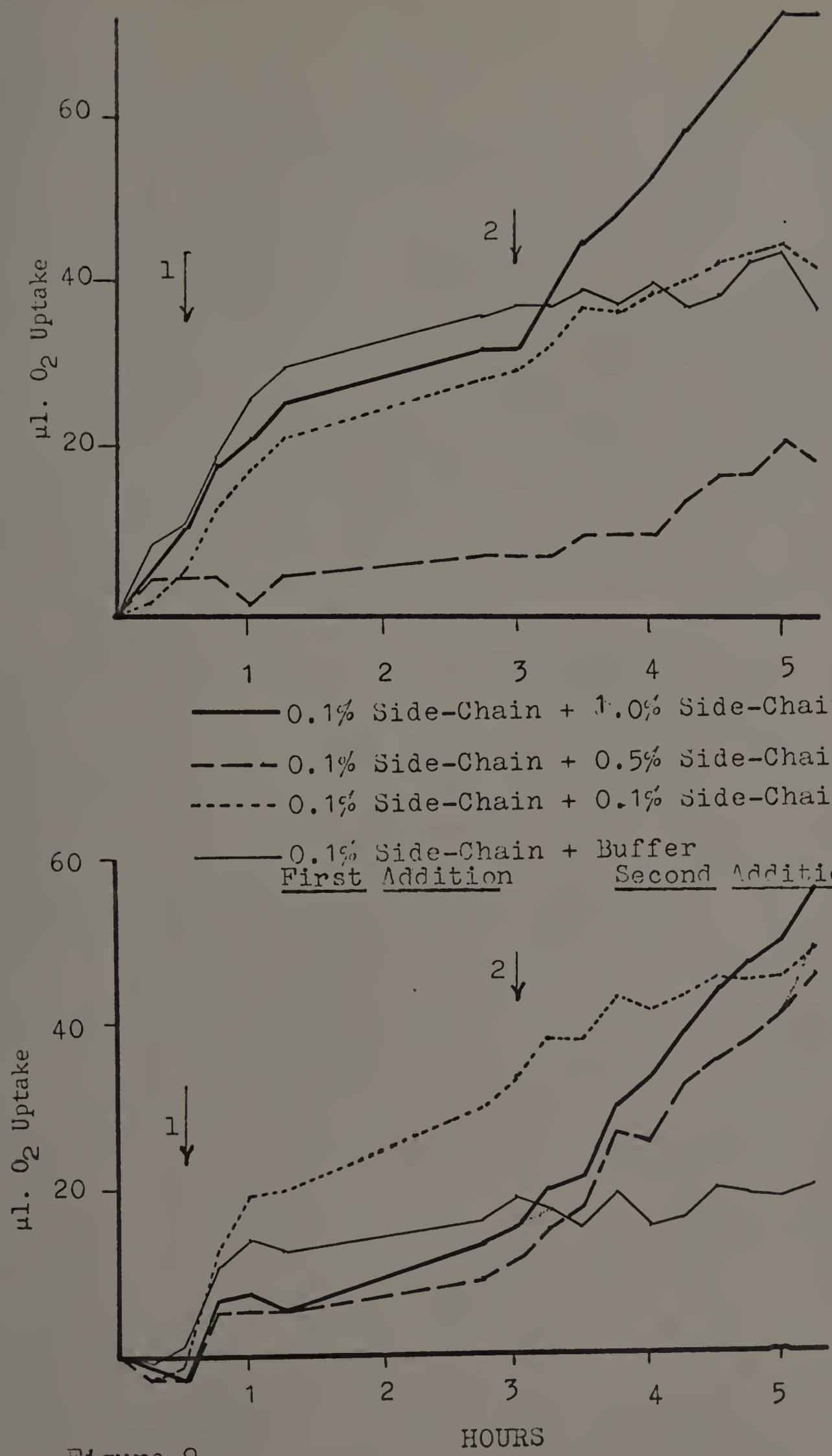
Figure 8
Metabolism of Diazinon side-chain as a function of side-chain concentration.

uptake with increased side chain concentration.

A study was undertaken of side chain utilization in the absence of glucose. The substrate was added in two increments, so that a diauxie reaction might be observed. Both isolates were grown 56 hours in minimal salts medium with 1% glucose, harvested, washed three times, starved for 5 days, harvested again, and resuspended in sterile buffer. The flasks were set up as follows, the first five in duplicate: the center well of each vessel was filled with 0.2 ml of 20% KOH.

| Center Vessel | Side Arm No. 1 | No. 2 |
|---|---|---|
| cells | substrate 1 ml | substrate 1 ml |
| 1 ml | buffer | buffer |
| 1 ml | side chain-1 | buffer |
| 1 ml | side chain-1 | side chain-1 |
| 1 ml | side chain-1 | side chain-2 |
| 1 ml | side chain-1 | side chain-3 |
| buffer 1 ml | side chain-1 | side chain-1 |
| buffer 1 ml | side chain-1 | side chain-3 |

Side chain concentrations were: 0.1% side chain-1, 0.5% side chain-2, 1.0% side chain-3. Contents of the first side arm were added 30 minutes after the initial readings were taken. The second side arm contents were added 150 minutes later. The data obtained are illustrated in Figure 9. A definite diauxie reaction is indicated with side chain-3 with isolates 9 and 16. And there is a striking resumption of $O_2$ uptake at the maximum concentration of side chain. This would indicate that the cells




No. 9

No. 16

Figure 9

Metabolism of Diazinon side-chain in the absence of glucose.

have been fully adapted for the metabolism of the substrate and could resume the activity with no lag phase. Of equal significance is the increase in the rate of side chain metabolism upon the addition of the second increment. The change in slope of the $O_2$ uptake curve for each isolate indicates that it is now capable of metabolizing a larger increment of side chain per unit time. Further, the degree of change in slope is a direct reflection of the concentration of the second increment of Diazinon side chain added.

When side chain concentration was increased to 5%, the most dramatic increase in $O_2$ uptake occurred with the addition of a second increment. The response of the isolates to the range including this concentration is shown in Figure 10. The same relationship between the slope of $O_2$ uptake and substrate concentration prevailed. The no-cell control again confirmed that chemical action on the side chain alone was not responsible for $O_2$ uptake.

In the final study of this series, it was proposed to examine the $CO_2$ evolution in relation to the $O_2$ uptake. Isolates nos. 9 and 16 were grown 30 hours in minimal salts medium with 1% glucose, harvested, washed three times, starved for 2 days, and harvested again. Following resuspension of the cells, the flasks were filled in duplicate as indicated:

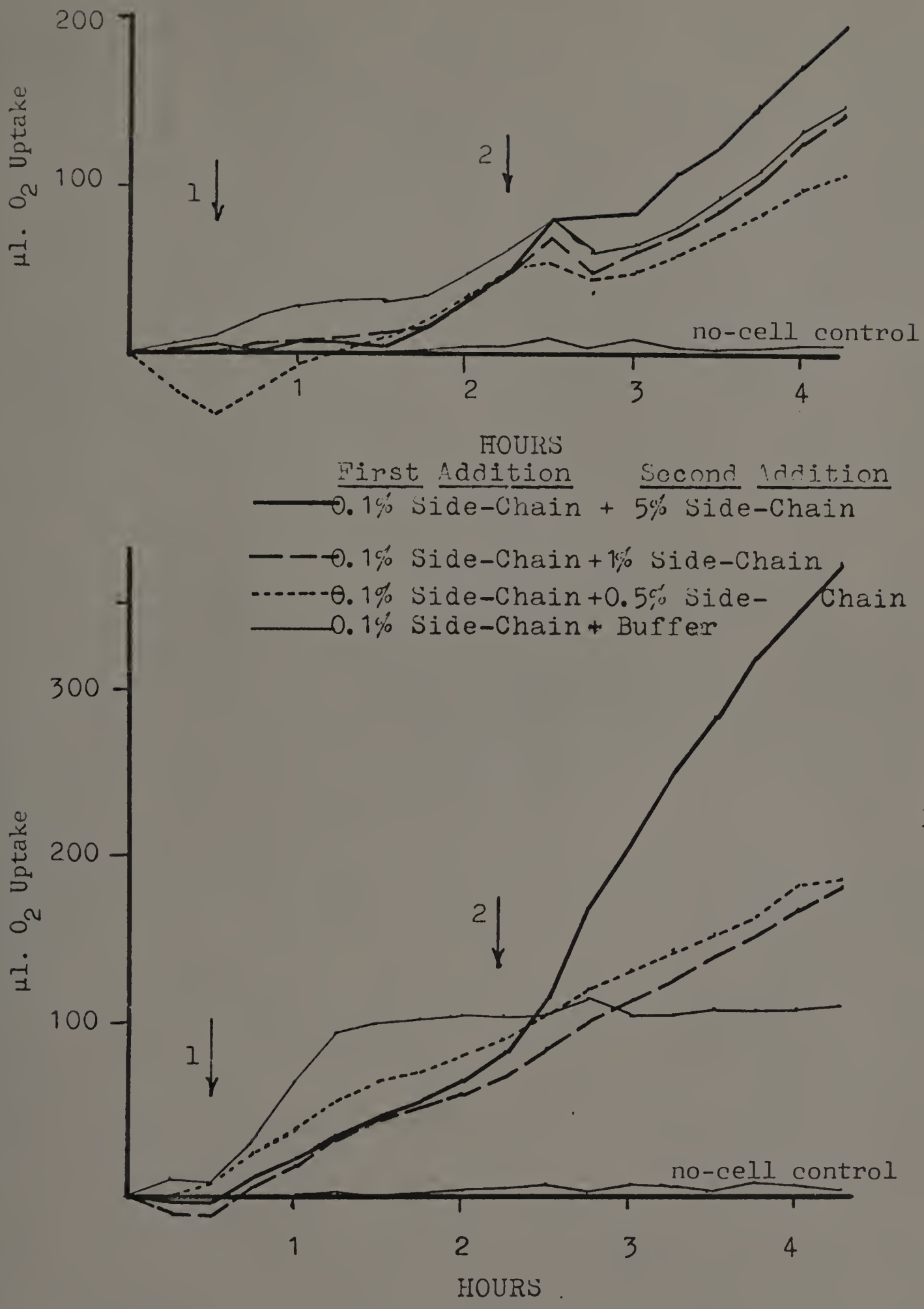



Figure 10
Effect of higher concentrations of Diazinon side-chain on the microbial metabolism of the side-chain.

| Center Well # | Center Vessel | Side Arm no. 1 cells | No. 2 5N $H_2SO_4$ |
|---|---|---|---|
| 1 0.2 ml | buffer 2.0 ml | 1.0 ml | -- |
| 2 -- | buffer 1.0 ml | 1.0 ml | 1.0 ml* |
| 3 -- | buffer 1.0 ml | 1.0 ml | 1.0 ml |
| 4 0.2 ml | buffer 1.5 ml side chain 0.5 ml | 1.0 ml | -- |
| 5 -- | buffer 0.5 ml side chain 0.5 ml | 1.0 ml | 1.0 ml* |
| 6 -- | buffer 0.5 ml side chain 0.5 ml | 1.0 ml | 1.0 ml |

Side chain concentration was 1.0%. Readings were taken for one-half hour, at which time the contents of the first side arm were poured into the center vessel; at the same time, contents of side arm no. 2 of those flasks starred (*) were also introduced. After 3.5 hours, $H_2SO_4$ was poured into the center vessel of those flasks still containing acid in the second side arm. The results of this experiment are shown in Figure 11. $CO_2$ determination was by the method described by Umbreit et al. (73). The data obtained are presented in Table 5. From these data, it would appear that the $O_2$ consumed was primarily in the oxidation of the ethyl ester carbons.

B. Detection of metabolites in the degradation of Diazinon side chain. In the investigation of optimal conditions for applying thin-layer chromatography to the detection of side chain metabolites, six solvent systems were initially studied, using microscope slides coated with

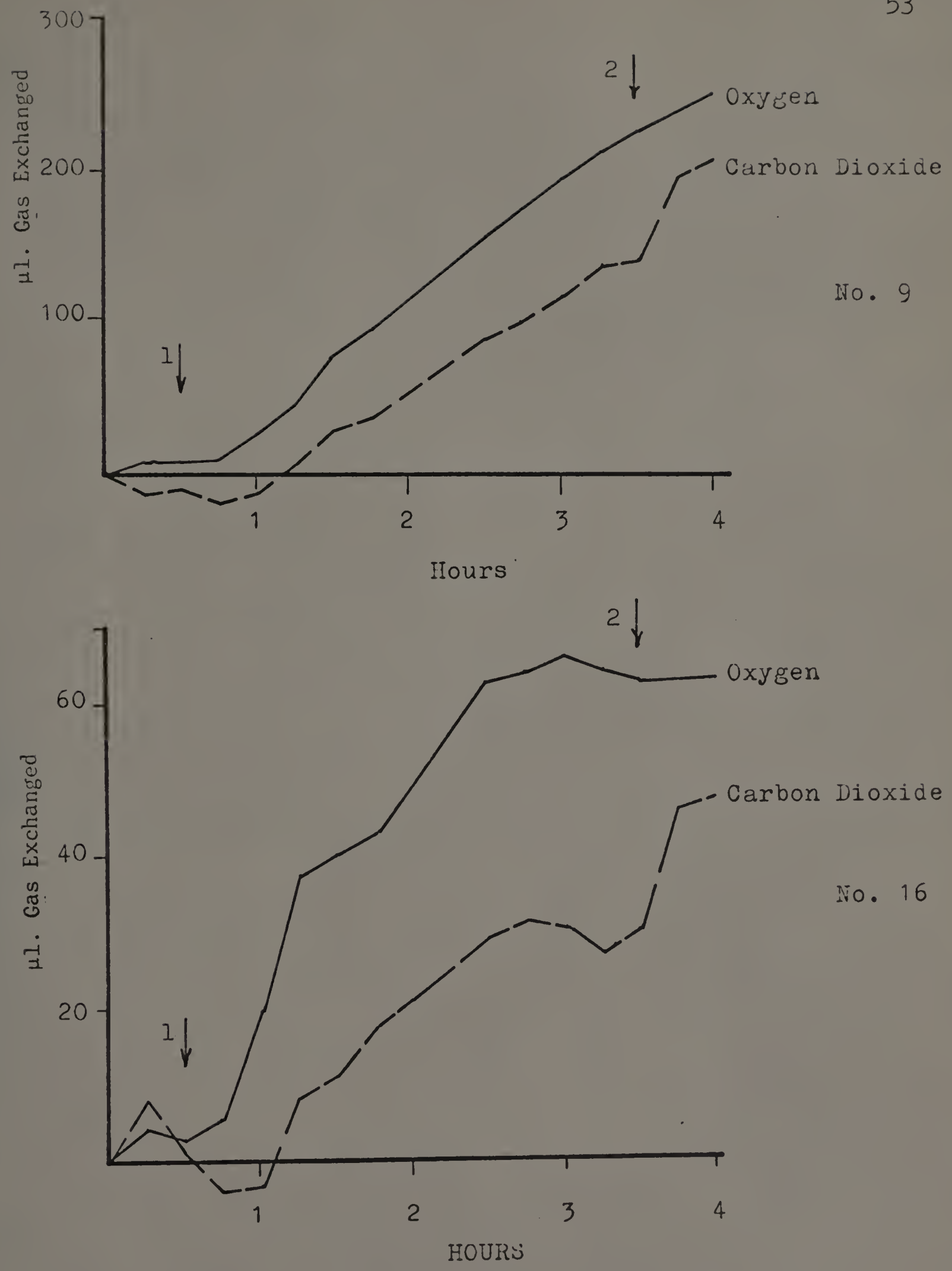



Figure 11
Respiration of isolates metabolizing Diazinon side-chain.

TABLE 5. Respiration of soil isolates metabolizing Diazinon side chain.

| | Isolate no. 9 | No. 16 |
|---|---|---|
| Endogenous $O_2$ Uptake | 256.2 µl | 337.6 µl |
| Endogenous $CO_2$ Evolution | 225.0 | 280.2 |
| Endogenous $CO_2/O_2$ | 0.88 | 0.83 |
| *Substrate $O_2$ Uptake | 250.7 µl | 63.5 µl |
| *Substrate $CO_2$ Evolution | 207.8 | 57.8 |
| *Substrate $CO_2/O_2$ | 0.83 | 0.91 |

*Corrected for endogenous values.

Adsorbosil-1. The side chain molecule reacted as follows: Solvent System (1) chloroform-methanol 9:1, no migration from point of application; Solvent System (2) chloroform-methanol 5:5, a streak along the upper half of the slide; Solvent System (3) methanol, no side chain detected; Solvent System (4) n-butanol-pyridine-water 6:4:3, migration was about 5/6 of the solvent front -- the spot was small; Solvent System (5) methanol-methylene chloride-ammonia (10%) 20:80:3, migration was less than 1/2 the solvent front, but the spot was more diffuse than (4); Solvent System (6) n-hexane-acetone 8:2, no migration. In a further investigation of solvent systems, the following systems were also studied, with the results as indicated: Solvent System (7) butanol-pyridine-water 6:4:3, $R_f$ about 5/6, spot was smallest of this series; Solvent System (8) B-P-W 7:2:1.5, $R_f$ 3/4, lateral spreading of spot; Solvent System (9) ethyl acetate-acetic acid-water 6:2:6, $R_f$ 1/2, lateral spreading and tailing of spot; Solvent System (10) butanol-acetic acid-water 12:3:5, $R_f$ 4/5, less tailing than (3). The B-P-W system was studied to find the ratio of components that would give a small spot and an $R_f$ of about 1/2. The combinations used and the results were: (1) B-W 2:8, $R_f$ 3/4, lateral spreading of spot; (2) B-W 8:2, $R_f$ 2/5, large spot; (3) B-P-W 2:9.5:8, $R_f$ 3/4, small, cross-shaped spot; and (4) B-P-W 8:0.5:2, $R_f$ 1/2, smallest spot of the four.

Using the B-P-W system 8:0.5:2, a chromatographic

analysis was made of the supernatant fluid of representative Warburg reaction vessels, as well as of standard solutions of Diazinon and Diazinon side chain. After spraying with the $(NH_4)_2PdCl_4$ solution for the P=S group, only one spot was seen for each spot applied. The $R_f$ of Diazinon was more than twice that of the side chain. The lowest concentration of side chain detected was 3 μg. A similar chromatogram was made to test the sensitivity of the detection system of Wade and Morgan (78). In order to provide a comparison for the Wade and Morgan (78) spray, the plate was so spotted that both halves of the plate contained the identical spots. One-half of the plate was covered with a piece of glass, while the other half was sprayed according to the procedure of Wade and Morgan (78). Then, the half already sprayed was covered with the glass, and the other half was sprayed with the $(NH_4)_2PdCl_4$ solution. Thus, the sensitivity of the Wade and Morgan (78) spray could be compared with that of the $(NH_4)_2PdCl_4$ spray. When the chromatogram was sprayed according to Wade and Morgan's (78) procedure, the area sprayed turned a lavender color, as was expected. The only white spots were found at the point of application of the Warburg supernatant fluids. The $(NH_4)_2PdCl_4$ spray revealed one spot for each point of application. No color appeared at the origin, but in positions similar to those described for the previous chromatogram.

The next experiment in thin-layer chromatography was

designed to compare the ability of the modified Hanes-Isherwood spray to detect the side chain with that of $(NH_4)_2PdCl_4$. In addition to Diazinon and side chain standards, Warburg supernatant fluids and lecithin were applied. The double half-plate procedure was repeated. The $(NH_4)_2PdCl_4$ solution detected the presence of Diazinon and side chain, but neither the buffer nor the lecithin, whereas the Hanes-Isherwood spray detected the presence of the side chain at the same location as had the $(NH_4)_2PdCl_4$ and, in addition, detected the buffer (not migrated from application point), as well as the lecithin. Again, the $R_f$ of Diazinon was somewhat greater than twice the $R_f$ of the side chain.

A substitute for the toxic B-P-W system was tried: n-butanol-glacial acetic acid-water 60:15:25. Following the double half-plate procedure, the plate was developed and sprayed with the $(NH_4)_2PdCl_4$ and Hanes-Isherwood sprays. Results obtained were similar to previous ones, but, in addition, the Hanes-Isherwood spray revealed a second spot behind some of the side chain spots with an $R_f$ of about 1/3 of the side chain. Material from Warburg supernatant fluids gave rise to these two spots. In order to ascertain whether the second spot was an artifact, another thin-layer chromatogram was prepared which included Diazinon and side chain standards, Warburg flask supernatant fluids, and phosphate buffer. Following development and spray application, it became apparent that the second spot (detected

on the previous chromatogram) corresponded to the spot of the phosphate buffer. Thus, no metabolites of the side chain appeared in any of the test systems. In this final chromatogram, another spray for the detection of phosphate was used. The spray, by Dittmer, however, only detected side chain concentrations greater than 105 µg.

A method of quantitating phosphorothioate concentration has been described by Akerfeldt and Lovgren (3). Standard curve determination was conducted in triplicate, according to the procedure previously described. The graph of the standard curve obtained is represented in Figure 12.

Preliminary studies indicated that there was little loss of the P=S group during Warburg respirometry studies. This was consistent with the lack of detectable metabolites other than intact side chain by thin-layer chromatography.

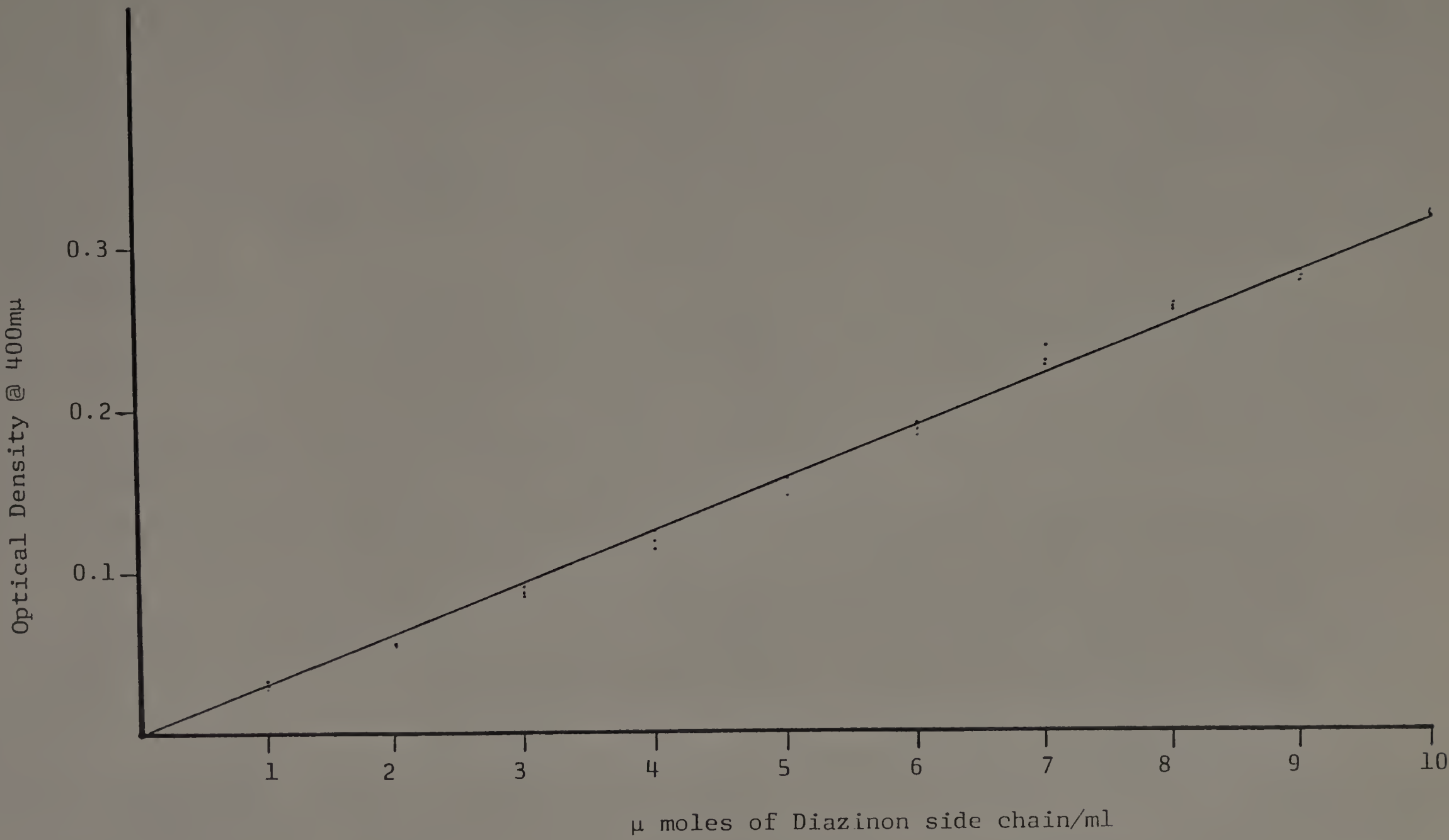



Figure 12 Standard curve for side chain determination by the method of Ackerfeldt and Lovgren.

# DISCUSSION

## I. The response of the microflora to the presence of Diazinon

The composition of the soil is extremely complex from both a chemical and a biological point of view. Neither the chemical nor the biological composition of the soil is stable. An equilibrium exists in both and between both. Should the equilibrium be disrupted, processes develop which ultimately result in the re-establishment of the equilibrium. These considerations are reflected in the varied results reported on the response of the soil microflora to applications of Diazinon and other organophosphates. Sideropoulos and his co-workers found that subsequent to a Diazinon application of 10 pounds per acre or greater, bacterial numbers increased as did the evolution of soil $CO_2$ (66).

The results of a similar investigation confirmed these findings (41). Of special interest was the soil microflora obtained from soil enrichment studies with Diazinon which resulted in the selection of bacteria capable of producing large quantities of polysaccharide-slime with the exclusion of most other bacteria (71). On the other hand, the study conducted by Bartha, Lanzilotta, and Pramer showed that the application of organophosphates to the soil resulted in an initial increase and subsequent decrease in soil $CO_2$ produc-

tion (8). In the study reported here, a significant decrease in bacterial numbers occurred.

The discrepancies in these findings may be explained in that the microfloral responses to Diazinon vary with time. Bartha's observations were made over a period of 30 days; Sideropoulos made no mention of time in his study. In the other investigations cited (41,71), observations ended at 90 days, whereas the data here reported concerned a period of 150 days. Thus, these data may represent microfloral responses at various stages in the process of equilibrium reestablishment after disruption by the addition of Diazinon. It might be speculated that the presence of Diazinon follows a line dictated by its biochemical potential. Thus, as the spectrum of activity of organophosphorus compounds is not limited to cholinesterase inhibition, but includes the inhibition of enzymes possessing carboxyesterase activity (65), the initial effect of Diazinon on at least a portion of the soil microflora could be bactericidal, as essential carboxyesterases of the susceptible population become inactivated. Subsequent to the elimination of the susceptible population, the number of microorganisms not susceptible to Diazinon increases, since there is now less competition for available substrates. Ultimately, from this selected population, tolerant of the present concentration of Diazinon and its residues, there arises a population of bacteria not only tolerant of

Diazinon, but capable of utilizing at least a portion of the Diazinon molecule as substrate. Such an organism would have a competitive advantage over the remainder of the microflora, being capable of metabolizing one more available substrate than the others. The slime, mentioned in the study reporting such a climax population to be predominantly composed of bacteria that produce large amounts of polysaccharide or slime, may have served as the mechanism by which the organisms solubilized the molecule or eliminated that portion of Diazinon metabolites that they were incapable of utilizing any further.

Such a climax population, however, would be able to maintain its predominance only as long as the Diazinon remained available. Once the Diazinon level dropped sufficiently, the heretofore suppressed population could once again enter the competition for available substrates, and the competitive advantage enjoyed by this climax population would become lost. On the basis of such renewed competition, an organism producing copious amounts of slime would be at a distinct competitive disadvantage. Consequently, the numbers of bacteria in this climax population would fall off rapidly. It might, therefore, be conjectured that the present study of the soil microflora was conducted at about the same time the specialized climax population no longer had available its special substrate and, lacking its competitive advantage, rapidly decreased in numbers. Had a

survey of the soil microflora been conducted still later, it may have been found that the total population had once again increased, approaching that equilibrium disrupted by Diazinon initially. Such a further investigation would appear merited.

II. Metabolism of the diethyl phosphorothioate moiety of the Diazinon molecule

That a portion of the Diazinon molecule was utilized was apparent from the initial Warburg study of the isolates obtained. Further refinement in the Warburg procedure showed that once the endogenous $O_2$ consumption was lowered, the diauxie effect became apparent. It was significant that, in general, the degree of response, as indicated by $O_2$ uptake, was in direct proportion to the amount of side chain added. This effect was evident at all three concentrations of glucose used. These results, however, did not entirely rule out the possibility that the diauxie reaction was achieved through a stimulatory effect of the side chain on glucose metabolism, i.e., the greater the side chain concentration, the stronger the stimulatory effect on glucose metabolism. Such an explanation was virtually eliminated in a later study in which both portions of the diauxie reaction were produced by side chain metabolism.

Again, it was noted that increased $O_2$ uptake by the isolates corresponded to the concentration of the side chain. Although the response to increased concentration of

side chain was not consistently linear, in general, the slope of $O_2$ consumption directly corresponded to the concentration of the side chain.

The fate of the $O_2$ consumed demands consideration. The oxygen could be involved in the oxidation of the phosphorothioate to phosphate, as has been mentioned (17,35,45,49, 55,58,62,64,68,74). Or, it may be consumed in the oxidation of the ethyl portion of the side chain molecule; incubation of an organism in the presence of $^{14}C$-ethyl Diazinon resulted in the recovery of $^{14}CO_2$, indicating the metabolism of at least the ethyl portion of the Diazinon molecule (34). The calculation of the $CO_2/O_2$ ratio of the organisms metabolizing the side chain should permit the assessment of which of the two side chain metabolism mechanisms is involved.

From the data in Table 5, it was apparent that a portion of the ethyl groups was metabolized to $CO_2$. However, the mechanism by which the ethyl groups were converted to $CO_2$ was not clear, judging by the experimental $CO_2/O_2$ values in the table. The $CO_2$ may have arisen by way of either of the pathways shown below:

$$\text{(I)}\quad CH_3CH_2OH + 3O_2 \longrightarrow 2CO_2 + 3H_2O$$

$$\text{(II)}\quad CH_3CH_2OH + 1\text{-}1/2O_2 \longrightarrow 2CO_2 + 6H^+$$

If equation (I) represented the main pathway for converting the ethyl groups to $CO_2$, the $CO_2/O_2$ ratio would be approximately 0.67. The values obtained were 0.83 and 0.91. If

equation (I) represented the pathway by which the $CO_2$ actually was evolved, there was more $CO_2$ evolved than could be accounted for by $O_2$ consumption. From this, it may be implied that either there was a carbon source other than the side chain, and in a more highly oxidized state, or there was some internal oxidant. That there was another carbon source, in a highly oxidized state, is unlikely, as the cells had been washed three times in phosphate buffer, starved for two days, harvested and resuspended in buffer. There are few readily available sources of oxygen other than the air. It may have been that the oxygen came from water or an inorganic or organic peroxide. However, to strip both hydrogens of a water molecule from the oxygen atom is an extremely difficult task; as the organisms, grown and starved aerobically, undoubtedly had an active peroxidase or catalase, it would be unlikely there would be any peroxides present. It, therefore, becomes exceedingly difficult to account for the $CO_2/O_2$ ratio obtained by equation (I).

On the other hand, the $CO_2/O_2$ ratio of equation (II) is 1.3. The experimental $CO_2/O_2$ ratios obtained fail to coincide with this theoretical ratio, also. The experimental ratios indicate too little $CO_2$ evolved for the $O_2$ consumed. Either not all of the carbon from the side chain molecule was converted to $CO_2$, or else some of the $O_2$ was taken up and used in some reaction other than oxidation of the ethyl

groups of the side chain. It is generally considered that production of cellular material consumes equal amounts of carbon, hydrogen, and oxygen, i.e., the formula for cellular material is represented by (CHO). If some of the side chain carbon went to cellular material production, an equal amount of oxygen would also be consumed. Thus, the $CO_2/O_2$ ratio would not be altered. There is one other possibility in which the consumption of $O_2$ can be accounted for without the production of $CO_2$. There is sufficient evidence for the enzymatic oxidation of the phosphorothioate moiety to phosphate (references, page 64), to postulate that some of the oxygen consumed by the isolates was involved in phosphorothioate oxidation. Consequently, the $CO_2/O_2$ ratios would be lower than if all of the $O_2$ consumed was used in the production of $CO_2$ according to equation (II). The elimination of the hydrogen ions, produced in equation (II), would not be difficult, since the entire Warburg reaction was carried out in a phosphate buffer solution. Thus, it may be concluded that the microbial metabolism of the O,O-diethyl phosphorothioate involves the oxidation of the phosphorothioate moiety to phosphate, as well as an assimilation and/or an oxidation of the ethyl groups to $CO_2$.

Several procedures were employed for the detection of metabolites of the side chain: thin-layer chromatography, colorimetric analysis, and enzyme assay. The first has been the most extensively developed of the three. The

buffer system used in these experiments was found to interfere with the detection methods that have been devised. Initial studies utilizing thin-layer chromatography and a colorimetric assay sensitive to the phosphorothioate group indicated that either the Diazinon side chain molecule was detected intact or was completely degraded to its individual constituents. Preliminary studies with an enzyme assay for ethanol suggested that only a very small amount of the side chain was chemically hydrolyzed, releasing ethanol. Further studies on the metabolic fate of the sulfur atom and the phosphate group are necessary.

The results of these studies indicate that microbial populations arising in the soil in response to Diazinon applications are capable of degrading the active side chain moiety of this molecule. The fate of the resulting fragments of decomposition, either released into the environment or integrated into microbial tissues, represents the logical extension of these studies.

# SUMMARY

The generally toxic properties of organophosphate compounds have been known since the early 1940's. However, little or no information has been available as to the microbial response to these compounds. Although the organophosphate, Diazinon, (0,0-diethyl-0-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorothioate) is extensively applied to soils, few studies have been reported with respect to its impact on the soil microflora or its biodegradability. The studies reported were designed to identify microbial response to Diazinon and to clarify the microbial capacity to degrade the active side chain portion of the Diazinon molecule.

Dilution plate studies indicated that in greenhouse soils, Diazinon exercised a remarkable and lasting suppressive effect on the bacterial population extending to 150 days subsequent to application.

Respirometric studies of the metabolism of the phosphorothioate side chain of Diazinon indicated that side chain utilization was a function of microbial growth. The presence of an exogenous carbon source was found to enhance side chain utilization. Different bacterial isolates from Diazinon-treated soils showed highly individual capacities for Diazinon side chain dissimilation. In adapted strains, dissimilation rates were directly propor-

tional to the concentration of Diazinon side chain present as substrate.

Respiratory quotient calculations indicated that whereas the major fraction of oxygen consumed was in the oxidation of the carbon atoms of the side chain, it could be inferred that the labile sulfur atom was oxidized as well. Thin-layer chromatographic analysis failed to show the presence of recognizable metabolites of Diazinon side chain, indicating the complete oxidation of the carbon atoms to $CO_2$ and the possible incorporation of the phosphorus and sulfur as part of the cell constituents.

# APPENDIX

## I. Media

### A. Modified Morris Medium

| Substance | | Amount per liter |
|---|---|---|
| $K_2HPO_4$ | | 7.0 g |
| $KH_2PO_4$ | | 3.0 g |
| $KNO_3$ | | 2.0 g |
| $MgSO_4 7H_2O$ | | 0.2 g |
| $FeCl_3$ (1% solution) | | 0.1 ml |
| $CaCl_3$ (1% solution) | | 0.5 ml |
| $MnCl_2 \cdot 4H_2O$ | $10^{-3}$ M | 0.2 ml |
| $H_3BO_3$ | $10^{-3}$ M | |
| $Na_2MoO_4$ | $10^{-3}$ M | 0.2 ml |
| $ZnSO_4 \cdot 7H_2O$ | $10^{-3}$ M | |
| $CuSO_4 \cdot 5H_2O$ | $10^{-3}$ M | 0.2 ml |
| $CoCl_2 \cdot 6H_2O$ | $10^{-3}$ M | 0.2 ml |
| Glucose 50% solution, sterilized separately | | 20.0 ml |

### B. Soil Extract Agar

| Substance | Amount per liter |
|---|---|
| Glucose | 1.0 g |
| $K_2HPO_4$ | 0.5 g |
| $KNO_3$ | 0.1 g |
| *Soil Extract | 100.0 ml |
| Agar | 18.0 g |

*Made by mixing 1000 g of soil with 1 liter of water and autoclaving the mixture for twenty minutes. About 0.5 g of calcium carbonate is added to precipitate colloidal material; the solution is filtered, the extract being the filtrate.

II. Sprays Used in Thin-Layer Chromatography

A. Ammonium Palladium Chloride

0.3% $(NH_4)_2PdCl_4$ in 1 N HCl.

B. DBCQ

0.5% 2,6-Dibromo-N-chloro-p-benzoquinoneimine in cyclohexane, heat at 100°C for 5 minutes, spray with 1 N HCl.

C. Salicylsulfonic Acid Spray

2 solutions: spray with solution (a), air dry, and spray with solution (b).

(a) 0.1% $FeCl_3$ in 80% ethanol.

(b) 1.0% salicyclsulfonic acid in 80% ethanol.

D. Hanes-Isherwood Phosphate Spray (modified)

10 ml 72% w/w perchloric acid
20 ml 5 N HCl
40 ml 5% $NH_4MoO_4$
130 ml $H_2O$

Develop under strong ultraviolet light.

E. Dittmer's Phosphate Spray

Solution (a) 40.11 g $MoO_3$ in 1 L 25 N $H_2SO_4$, boil until dissolved.

(b) 1.78 g powdered molybdenum in 500 ml of solution (a), boil gently 15 minutes, cool and decant.

The spray is made by mixing equal volumes of (a) and (b) and the resulting mixture with 2 volumes of water.

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